The ISME Journal (2012) 6, 577–587

www.nature.com/ismej

ORIGINAL ARTICLE

Reductive genome evolution, host–symbiont co-speciation and uterine transmission of endosymbiotic bacteria in bat flies

Takahiro Hosokawa[1,4], Naruo Nikoh[2,4], Ryuichi Koga[1,4], Masahiko Satô[3], Masahiko Tanahashi[1], Xian-Ying Meng[1] and Takema Fukatsu[1]

[1]*National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan;* [2]*Department of Liberal Arts, The Open University of Japan, Chiba, Japan and* [3]*Rishiri Town Museum, Rishiri, Japan*

Bat flies of the family Nycteribiidae are known for their extreme morphological and physiological traits specialized for ectoparasitic blood-feeding lifestyle on bats, including lack of wings, reduced head and eyes, adenotrophic viviparity with a highly developed uterus and milk glands, as well as association with endosymbiotic bacteria. We investigated Japanese nycteribiid bat flies representing 4 genera, 8 species and 27 populations for their bacterial endosymbionts. From all the nycteribiid species examined, a distinct clade of gammaproteobacteria was consistently detected, which was allied to endosymbionts of other insects such as *Riesia* spp. of primate lice and *Arsenophonus* spp. of diverse insects. In adult insects, the endosymbiont was localized in specific bacteriocytes in the abdomen, suggesting an intimate host–symbiont association. In adult females, the endosymbiont was also found in the cavity of milk gland tubules, which suggests uterine vertical transmission of the endosymbiont to larvae through milk gland secretion. In adult females of *Penicillidia jenynsii*, we discovered a previously unknown type of symbiotic organ in the Nycteribiidae: a pair of large bacteriomes located inside the swellings on the fifth abdominal ventral plate. The endosymbiont genes consistently exhibited adenine/thymine biased nucleotide compositions and accelerated rates of molecular evolution. The endosymbiont genome was estimated to be highly reduced, ~0.76 Mb in size. The endosymbiont phylogeny perfectly mirrored the host insect phylogeny, indicating strict vertical transmission and host–symbiont co-speciation in the evolutionary course of the Nycteribiidae. The designation '*Candidatus* Aschnera chinzeii' is proposed for the endosymbiont clade.

The ISME Journal (2012) **6,** 577–587; doi:10.1038/ismej.2011.125; published online 22 September 2011
Subject Category: microbe–microbe and microbe–host interactions
Keywords: endosymbiont; nycteribiid bat fly; vertical transmission; adenotrophic viviparity; co-evolution

Introduction

Insects represent the majority of eukaryotic biodiversity in the terrestrial ecosystem (Grimardi and Engel, 2005). One of the factors that have driven the prominent diversification may be their ability to use nutritionally incomplete or difficult food resources, such as woody material that is hard to digest and contains low nitrogen, plant sap that contains little proteins and lipids, vertebrate blood that is deficient in B vitamins, etc. In many, if not all, of these cases, it has been shown that symbiotic microorganisms have important roles in compensating for such nutritional deficiencies. In termites, for example, intestinal protozoans and bacteria enable their hosts to digest cellulose, and some bacteria are involved in nitrogen fixation (Breznak and Brune, 1994; Ohkuma, 2003). In aphids, an endocellular bacterium *Buchnera aphidicola* synthesizes essential amino acids that are scarce in their plant sap diet (Douglas, 1998; Shigenobu *et al.*, 2000). In tsetse flies, an endocellular bacterium *Wigglesworthia glossinidia* provides B vitamins that are deficient in their blood meal (Nogge, 1981; Akman *et al.*, 2002).

The feeding habit on vertebrate blood has evolved in some 14 000 insect species representing 5 orders, namely Siphonaptera (fleas), Phthiraptera (lice), Diptera (mosquitoes and flies), Hemiptera (bedbugs and kissing bugs) and Lepidoptera (an exceptional case of nocturid moth *Calyptra eustrigata*) (Adams, 1999; Lehane, 2005). Some of the insects such as mosquitoes and fleas feed on vertebrate blood only at their adult stage mainly for gonad maturation and egg production, whereas their larvae live on different food sources. On the other hand, lice, tsetse flies, bedbugs and others depend on vertebrate blood as the sole food source throughout their life. Notably,

Correspondence: T Fukatsu, Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan.
E-mail: t-fukatsu@aist.go.jp
[4]The authors contributed equally to this work.
Received 30 May 2011; revised 3 August 2011; accepted 5 August 2011; published online 22 September 2011

the latter insect groups generally possess a highly developed endosymbiotic system, consisting of specialized cells called bacteriocytes for harboring specific microbes and symbiotic organs called bacteriomes constituted by bacteriocytes and other types of cells. These patterns suggest the pivotal necessity of microbial associates for such insects that are highly adapted to obligate blood-feeding lifestyles (Buchner, 1965; Bourtzis and Miller, 2003).

Members of the dipteran superfamily Hippoboscoidea are highly specialized ectoparasitic insects, encompassing four family-level taxa: tsetse flies feeding on mammalian blood (Glossinidae), louse flies associated with birds or mammals (Hippoboscidae) and bat flies obligatorily ectoparasitic on bats (Streblidae and Nycteribiidae) (McAlpine, 1989). Hippoboscoid species are generally associated with bacterial endosymbionts, whereas their microbial partners and their symbiotic organs are remarkably different between the families (Roubaud, 1919; Zacharias, 1928; Aschner, 1931; Buchner, 1965). Among them, the endosymbiotic system in tsetse flies, consisting of an obligate endosymbiont *W. glossinidia* in a midgut-associated bacteriome (Aksoy, 1995) and a facultative endosymbiont *Sodalis glossinidius* (Dale and Maudlin, 1999), is the best studied, reflecting their medical importance as trypanosome vectors (Aksoy, 2000; Aksoy and Rio, 2005). The complete genome sequences of *Wigglesworthia* and *Sodalis* have already been determined (Akman *et al.*, 2002; Toh *et al.*, 2006). By contrast, much less attention has been paid to the endosymbiotic systems of hippoboscids, streblids and nycteribiids (Dale *et al.*, 2006; Trowbridge *et al.*, 2006; Nováková *et al.*, 2009), except for early histological descriptions (Roubaud, 1919; Zacharias, 1928; Aschner, 1931; Buchner, 1965).

Bat flies of the family Nycteribiidae are obligate ectoparasites of bats with strikingly specialized morphological traits, including lack of wings, reduced head and eyes, dorsoventrally flattened thorax, dorsally inserted legs, spider-like appearance due to these morphologies and adenotrophic viviparity with a highly developed uterus and milk glands (Lehane, 2005; Dick and Patterson, 2006). On account of their extreme specialization for ectoparasitic and blood-feeding lifestyle, intimate endosymbiotic associations may be expected for nycteribiid bat flies. However, previous works on the subject are quite limited. Aschner (1931) histologically inspected three nycteribiid species, namely *Nycteribia biarticulata*, *Nycteribia blassi* and *Nycteribia blainvillei*, and observed the presence of endosymbiotic bacteria in bacteriocytes that formed loose clusters in the abdominal body cavity in both males and females, and also in the cavity of milk gland tubules in females. Aschner (1946) also inspected a distinct nycteribiid species *Eucampsipoda aegyptia*, wherein endosymbiotic bacteria were restricted to milk gland tubules. Recently, Nováková *et al.* (2009) provided the first molecular characterization of nycteribiid endosymbionts. Three nycteribiid species, *Nycteribia kolenati*, *Penicillidia monoceros* and *Penicillidia* sp., were subjected to cloning and sequencing of the bacterial 16S rRNA gene, which yielded two groups of gammaproteobacterial sequences: a group of sequences were placed within the clade of *Arsenophonus* spp. that are facultative endosymbionts associated with diverse insects; and another group of sequences formed a distinct clade, which exhibited phylogenetic affinity to the clade of *Riesia* spp., obligate endosymbionts of primate lice and also to the clade of *Arsenophonus* spp. (Nováková *et al.*, 2009). However, the early histological data and the recent molecular data are currently not connected to each other, and should be integrated into a coherent picture. It is obscure which of the bacterial clades detected from the nycteribiids correspond to the endosymbionts harbored in bacteriocytes and those located in the milk glands, respectively. Co-evolutionary aspects between the endosymbionts and their nycteribiid hosts are largely unknown and yet to be established.

In this study, we investigated Japanese nycteribiid bat flies, which represented 4 genera, 8 species and 27 populations, for their bacterial endosymbionts. We identified a distinct clade of gammaproteobacterial endosymbionts from all the nycteribiid species as reported previously (Nováková *et al.*, 2009), and also unveiled a number of previously unknown aspects in the nycteribiid–bacterium association, including the discovery of a pair of large bacteriomes in the abdomen of a *Penicillidia* species, detailed *in vivo* localization and ultrastructure of the endosymbiont, remarkable reductive evolutionary patterns in the endosymbiont genome and strict host–symbiont co-speciation.

Materials and methods

Insect materials

Nycteribiid bat flies were collected by bat ecologists in their field surveys and provided for this study (Supplementary Table S1; Supplementary Figure S1). Most of the samples were preserved in acetone upon collection for DNA analyses (Fukatsu, 1999). Some insects of *Penicillidia jenynsii* were anesthetized by chilling on ice, dissected and subjected to either *in situ* hybridization, electron microscopy or pulsed-field gel electrophoresis.

DNA extraction and morphological inspection

Acetone-preserved bat flies were individually subjected to DNA extraction and morphological inspection. Each fixed insect was dried in air, and its abdomen was detached using forceps under a dissection microscope. The insect parts were placed in a 1.5-ml plastic tube and digested in 200 μl of a proteinase K-containing lysis buffer supplied with

the QIAamp tissue mini kit (Qiagen, Venlo, The Netherlands) at 55 °C overnight. The lysate was subjected to DNA extraction using the kit, while the resultant exoskeleton was thoroughly washed with distilled water, stored in 70% ethanol and observed under a dissection microscope.

Cloning, genotyping and sequencing
Bacterial 16S rRNA, *groEL* and *gyrB* genes were amplified by PCR from the DNA samples, and subjected to cloning, restriction fragment length polymorphism (RFLP) genotyping using restriction endonuclease *HaeIII*, and DNA sequencing as described previously (Kikuchi *et al*, 2009). Insect mitochondrial *COI* (cytochrome oxidase I) and 16S rRNA genes were also amplified by PCR and subjected to direct sequencing. The primers and PCR conditions are summarized in Supplementary Table S2.

Molecular phylogenetic, molecular evolutionary and genomic analyses
Multiple alignments of the nucleotide sequences were generated using the program Clustal W (Thompson *et al*., 1994). Phylogenetic analyses were conducted by neighbor-joining, maximum parsimony and maximum likelihood methods, using the programs Clustal W (Thompson *et al*., 1994), PAUP 4.0b10 (Swofford, 2001) and Phyml (Guindon and Gascuel, 2003), respectively. Relative rate tests were performed using the program RRTree (Robinson-Rechavi and Huchon, 2000). Co-evolutionary analyses were performed under the jungles algorithm using the program TreeMap v2.02β, which evaluates the extent of topological congruence between phylogenies (Charleston and Page, 2002). Randomization tests were conducted as described previously (Hosokawa *et al*., 2006). Pulsed-field gel electrophoresis of the endosymbiont genome was performed with the bacteriome samples dissected from 50 adult females of *P. jenynsii*. The tissues were gently homogenized in 50 µl of phosphate-buffered saline containing 10 mM EDTA, and the homogenate was filtered through a 10-µm nylon mesh, and the filtered suspension was mixed with 1% low-melting point agarose. The agarose plug was cut into an appropriate shape and size, and treated with proteinase K at 50 °C overnight. After thorough washing, the plug was subjected to digestion with restriction endonuclease *I-CeuI*. Pulsed-field gel electrophoresis was conducted using CHEF Mapper XA (Bio-Rad, Hercules, CA, USA).

Histological procedures
Insects were dissected and fixed in Carnoy's solution (ethanol–chloroform–acetic acid, 6:3:1) overnight, and treated with 6% hydrogen peroxide in 80% ethanol for 2 days to reduce autofluorescence of tissues (Koga *et al*., 2009). The dissected tissues were thoroughly washed with 100% ethanol and then with phosphate-buffered saline, and subjected to *in situ* hybridization with fluorochrome-labeled oligonucleotide probes targeting 16S rRNA of the endosymbionts, Al555-BF440 (5′-TTAATKCCTTCCTCACAACTG-3′) and Al555-B F1256 (5′-TCCCATCGCTGGCTAGCCT-3′), as described previously (Kikuchi *et al*., 2009). For transmission electron microscopy, insects were dissected with fine forceps in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde, and isolated tissues were processed into ultra-thin sections and observed as described previously (Fukatsu *et al*., 2000).

Results

16S rRNA genes from nycteribiid bat flies P. jenynsii *and* Nycteribia allotopa
During an ecological monitoring of a natural bat population at Sayo-cho (Hyogo, Japan), two bat fly species, larger *P. jenynsii* and smaller *N. allotopa* (Figure 1; Supplementary Table S1), were collected from the Eastern bent-winged bats *Miniopterus fuliginosus*. Two individuals of *P. jenynsii* and two individuals of *N. allotopa* were subjected to DNA extraction and PCR amplification of bacterial 16S rRNA genes. PCR products were cloned, and 30 clones per insect were subjected to RFLP genotyping. All clones from *N. allotopa* exhibited an identical RFLP genotype, whereas clones from *P. jenynsii* consisted of a major frequent genotype and a minor infrequent genotype. For each of the RFLP genotypes, three clones per insect were sequenced. All sequences of the major genotype from *P. jenynsii* were 1464 bp in size and completely

Figure 1 Nycteribiid bat flies, *Penicillidia jenynsii* (top) and *Nycteribia allotopa* (bottom). Acetone-preserved specimens are shown.

identical to each other, exhibiting the BLAST top hit to 16S rRNA gene sequence of *Arsenophonus* endosymbiont from scale insect *Australiococcus greville* (92.6% (1358/1467) sequence identity; accession number AY264673). When ~1 kb region of the sequence corresponding to the sequenced region of the 16S rRNA gene reported by Nováková *et al*. (2009) was subjected to the BLAST search, the higher hit was identified as the 16S rRNA gene sequence of endosymbiont from nycteribiid bat fly *P. monoceros* (97.2% (1030/1060) sequence identity; accession number FJ265817). Meanwhile, all sequences from *N. allotopa* were 1473 bp in size and almost completely identical to each other, but a single nucleotide site between the two individuals, exhibiting the BLAST top hit to the 16S rRNA gene sequence of *Arsenophonus* endosymbiont from citrus psyllid *Diaphorina citri* (92.5% (1362/1473) sequence identity; accession number AB038366). When ~1 kb region of the sequence corresponding to the sequenced region of the 16S rRNA gene reported by Nováková *et al*. (2009) was subjected to the BLAST search, the higher hit was identified as the 16S rRNA gene sequence of endosymbiont from nycteribiid bat fly *N. kolenatii* (97.2% (1039/1069) sequence identity; accession number FJ265804). All sequences of the minor genotype from *P. jenynsii* were 1426 bp in size and identical to each other, exhibiting the BLAST top hit to the 16S rRNA gene sequence of *Wolbachia* endosymbiont from louse fly *Pseudolynchia cararіensis* (99.6% (1420/1425) sequence identity; accession number DQ115537).

Phylogenetic placement of bat fly endosymbionts

Figure 2 shows the phylogenetic placement of the endosymbionts of *P. jenynsii* and *N. allotopa* in the *Gammaproteobacteria* on the basis of their 16S rRNA gene sequences. The endosymbiont sequences formed a robust clade, supported by 100% bootstrap values, together with 16S rRNA gene sequences that had previously been obtained from other nycteribiid bat flies (Nováková *et al*., 2009). It was notable that the phylogenetic relationship of the bacterial sequences reflected the systematics of bat fly hosts: the sequences from *Penicillidia* spp., namely *P. jenynsii*, *P. monoceros* and *P.* sp., formed a clade, whereas the sequences from *Nycteribia* spp., namely *N. allotopa* and *N. kolenatii*, constituted a sister clade to it. Adjacent to the endosymbiont clade of the nycteribiid bat flies, endosymbionts of primate lice, *Riesia* spp., formed a sister clade. Adjacent to them, endosymbionts of a hippoboscid fly *Lipoptena* sp. and streblid bat flies *Trichobius* spp. formed a clade. Allied to them was a clade of facultative *Arsenophonus* endosymbionts associated with diverse insects (Nováková *et al*., 2009). All these *Arsenophonus*-allied endosymbionts as a whole constituted a robust clade, supported by 100% bootstrap values, in the *Gammaproteobacteria*.

In vivo localization and fine structure of bat fly endosymbionts

To identify *in vivo* localization of bat fly endosymbionts, adult insects of *N. allotopa* and *P. jenynsii* were subjected to whole-mount *in situ* hybridization with fluorochrome-labeled probes specifically targeting the endosymbiont 16S rRNA.

In the abdomen of adult insects of *N. allotopa*, signals of the endosymbiont were detected in clusters of small bacteriocytes (Supplementary Figure S2). The localization patterns of bacteriocytes were similar to those previously observed in *N. biarticulata* and *N. blasii* (Aschner, 1931).

In the abdomen of adult males of *P. jenynsii* (Figure 3a), signals of the endosymbiont were detected in clusters of small bacteriocytes just

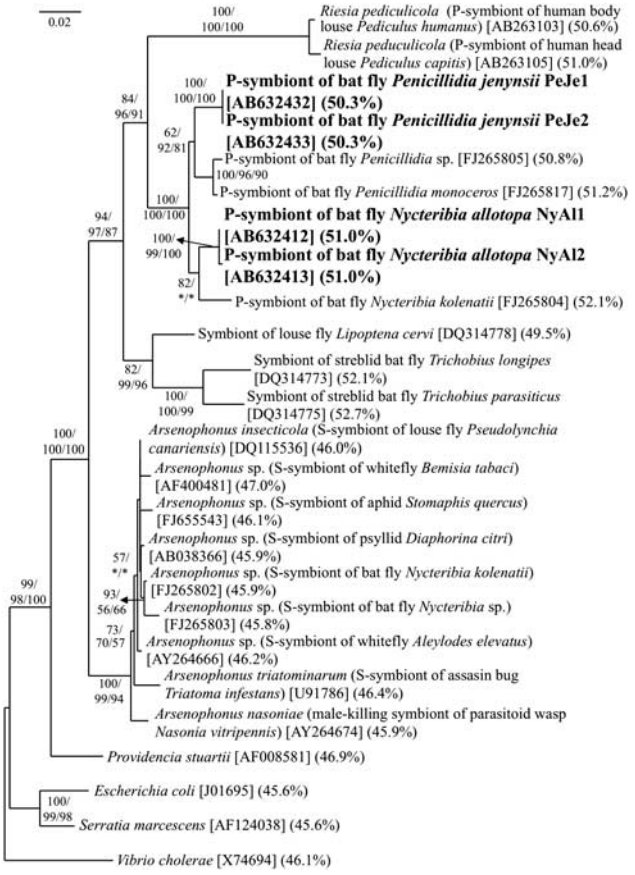

Figure 2 Phylogenetic placement of the endosymbionts of nycteribiid bat flies *Penicillidia jenynsii* and *Nycteribia allotopa* in the *Gammaproteobacteria* on the basis of 16S rRNA gene sequences. A neighbor-joining tree inferred from a total of 973 aligned nucleotide sites is shown, whereas maximum parsimony and maximum likelihood analyses produced substantially the same results. Bootstrap values no less than 50% are indicated at the nodes in the order of neighbor-joining/maximum parsimony/maximum likelihood, whereas asterisks indicate support values less than 50%. Sequence accession numbers and AT contents of the nucleotide sequences are in brackets and parentheses, respectively. As for insect symbionts, the information of the host insect may also be indicated in parentheses. P-symbiont, primary symbiont of prevalent infection and obligatory nature; S-symbiont, secondary symbiont of sporadic infection and facultative nature.

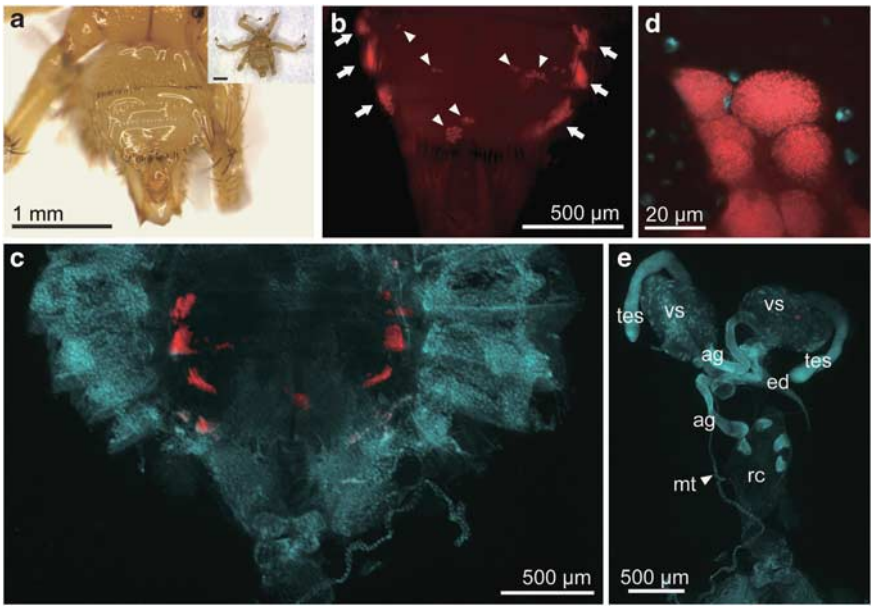

Figure 3 *In vivo* localization of the endosymbiont in adult males of *Penicillidia jenynsii*. (**a**) An external ventral view of the abdomen of an adult male. Inset is an image of the whole body. (**b–e**) Visualization of the endosymbiont by 16S rRNA-targeted fluorescent *in situ* hybridization. Red signals indicate the endosymbiont, whereas blue signals show host insect nuclei. (Panel **b**) A ventral view of the abdomen, wherein dense clusters of bacteriocytes are located on both sides of the third, fourth and fifth abdominal segments (arrows), and less dense clusters are also seen under the ventral plates (arrowheads). (Panel **c**) A ventral view of the dissected abdomen, the ventral plates of which are cut and opened. (Panel **d**) An enlarged image of the bacteriocytes. (Panel **e**) Dissected reproductive organs of a male, wherein few signals of the endosymbiont are seen. ag, accessory gland; ed, ejaculatory duct; mt, Malphigian tubule; rc, rectum; tes, testis; vs, vesicula seminalis.

beneath the cuticle, the most densely on both sides of the second, third, fourth and fifth abdominal segments and less densely under the ventral plates (Figures 3b and c). The localization patterns of bacteriocytes were a little different, but not so divergent from those observed in *Nycteribia* spp. (Aschner, 1931; see Supplementary Figure S2). The bacteriocytes were ~20 μm in diameter, their nucleus was located on the periphery of the cells, and their cytoplasm was filled with numerous endosymbiont cells (Figure 3d). In the male reproductive organs, few signals of the endosymbiont were detected (Figure 3e).

In the abdomen of adult females of *P. jenynsii* (Figure 4a), strikingly, the endosymbiont exhibited localization patterns quite different from those in males. In the female abdomen, the fifth ventral plate was well developed and equipped with a pair of swellings on both sides (Figure 4a, arrows). *In situ* hybridization identified very strong signals inside the swellings, where a pair of large bacteriomes consisting of a number of bacteriocytes was located (Figure 4b). The bacteriocytes were, like those in males, ~20 μm in diameter with a peripheral nucleus and full of numerous endosymbiont cells in the cytoplasm (Figure 4c). The abdominal cavity of adult females was mostly occupied by highly developed reproductive organs consisting of a uterus encasing a developing larva inside, a few immature oocytes, and highly branched milk glands for supplying food secretion to the developing larva (Figures 4d and e). Interestingly, signals of the endosymbiont were detected inside the milk gland tubules (Figures 4e and f), indicating the presence of endosymbiont cells in milk gland secretion.

Electron microscopy revealed numerous tubular bacterial cells packed in the cytoplasm of bacteriocytes (Figures 5a and b), and confirmed the presence of endosymbiont cells in milk gland secretion (Figure 5c).

Molecular phylogenetic analyses of nycteribiid bat flies

In an attempt to understand the evolution of the host–symbiont association in the Nycteribiidae, we collected 41 individuals of nycteribiid bat flies representing 4 genera, 8 species and 27 populations in Japan, and several endosymbiont genes and host insect genes were cloned and sequenced from them (Supplementary Table S1; Supplementary Figure S1). Here, we focused on the bacteriocyte-associated endosymbiont, and thus sequenced clones of the major RFLP genotype for each of the insect samples. On the basis of these sequence data, we conducted a series of molecular phylogenetic analyses.

Phylogenetic analyses of endosymbiont genes

Phylogenetic relationships of endosymbionts inferred from the nucleotide sequences of the 16S rRNA gene (Supplementary Figure S3), *gyrB* gene (Supplementary Figure S4) and *groEL* gene

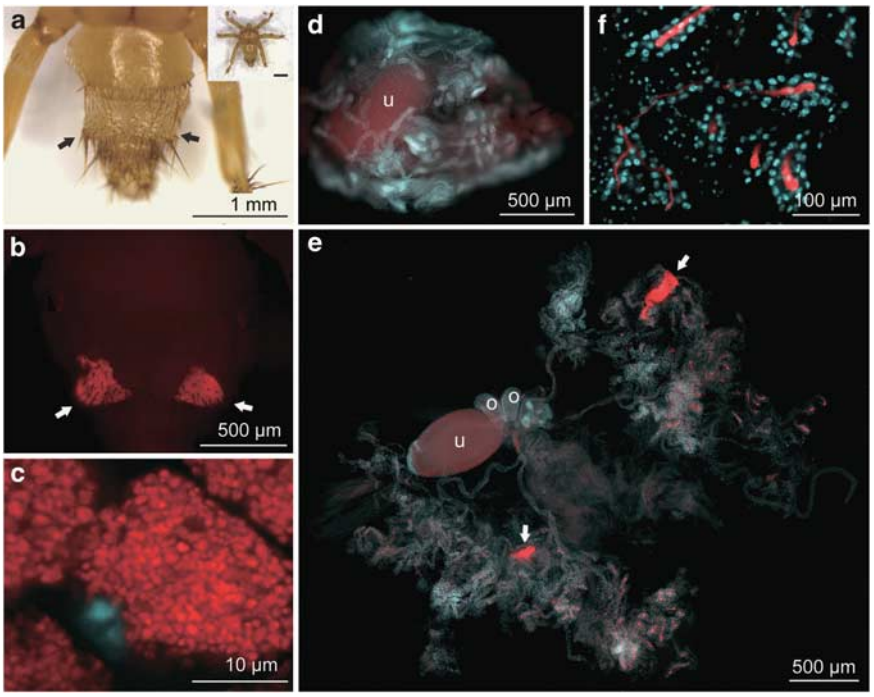

Figure 4 *In vivo* localization of the endosymbiont in adult females of *Penicillidia jenynsii*. (**a**) An external ventral view of the abdomen of an adult female. Arrows indicate swellings of the fifth ventral plate. Inset is an image of the whole body. (**b–f**) Visualization of the endosymbiont by 16S rRNA-targeted fluorescent *in situ* hybridization. Red signals indicate the endosymbiont, whereas blue signals show host insect nuclei. (Panel **b**) A ventral view of the abdomen, in which a pair of large bacteriomes are visualized inside the swellings of the fifth ventral plate (arrows). (Panel **c**) An enlarged image of bacteriocytes in the bacteriome. (Panel **d**) A female abdomen full of a uterus and entwined milk gland tubules. (Panel **e**) Dissected reproductive organs of a female, consisting of a large uterus, a few immature oocytes, as well as huge and highly branched milk glands. It must be noted that signals of the endosymbiont are seen inside the milk gland tubules. Arrows indicate the bacteriomes fragmented during dissection. Red color on the uterus is due to autofluorescence. (Panel **f**) An enlarged image of the milk gland tubules. o, oocyte; u, uterus.

(Supplementary Figure S5) consistently agreed with the systematics of the host insects. Supplementary Figure S6 shows the phylogenetic tree based on the combined sequence data of the three genes. The endosymbiont sequences from the same host species were completely or nearly identical to each other, and therefore sequences representing each of the host species formed a distinct monophyletic group. Moreover, the endosymbiont sequences from each of the same host genera, namely *Nycteribia*, *Basilia* and *Penicillidia*, constituted a well-supported monophyletic group.

Phylogenetic analyses of host insect genes

Phylogenetic relationships of the host insects inferred from the nucleotide sequences of the mitochondrial *COI* gene (Supplementary Figure S7) and the 16S rRNA gene (Supplementary Figure S8) reflected the host insect systematics. Supplementary Figure S9 shows the phylogenetic tree based on the combined sequence data of the two genes. Sequences of the same insect species were closely related to each other, and therefore sequences representing each of the insect species formed a distinct monophyletic group. Furthermore, the sequences from each of the same host genera, namely *Nycteribia*, *Basilia* and *Penicillidia*, constituted a well-supported monophyletic group.

Phylogenetic analysis of host–symbiont co-evolution in nycteribiid bat flies

Figure 6 contrasts the phylogeny of bat fly species and of their endosymbionts. Strikingly, the phylogenetic relationship of the host insects was almost in complete concordance with the phylogenetic relationship of their endosymbionts. Although the placement of *Phthiridium hindlei* was slightly different between the phylogenies, the bootstrap supports for it were extremely poor in the endosymbiont phylogeny. Given that the placement of *Phthiridium hindlei* was treated as multi-furcated, the endosymbiont phylogeny was perfectly congruent with the host phylogeny. On account of a total of 135 135 possible rooted tree topologies for 8 taxa, the chance that the symbiont tree will exactly match the host tree is expected to be $<0.001\%$. The congruence was so complete that the jungles algorithm identified only one reconstruction of the co-evolutionary history with six co-divergence events (Figure 6). A randomization test also confirmed that such a high level of phylogenetic congruence is quite unlikely to occur by chance ($P=0.002$).

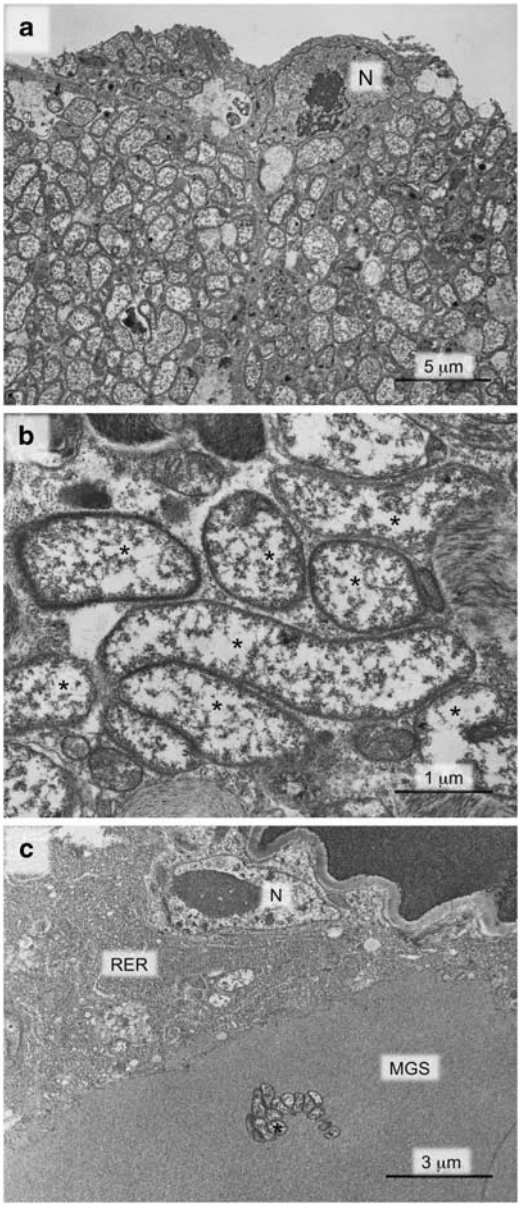

Figure 5 Transmission electron microscopy of the symbiotic organs in adult females of *Penicillidia jenynsii*. (**a**) Bacteriocytes in a female bacteriome, the cytoplasm is filled with endosymbiont cells. (**b**) An enlarged image of the endosymbiont cells. (**c**) An image of the milk gland epithelium with well-developed rough endoplasmic reticula, which is indicative of its high secretory activity, and the milk gland secretion containing endosymbiont cells in the inner cavity of the organ. In panels **b** and **c**, asterisks indicate endosymbiont cells. N, nucleus; MGS, milk gland secretion; RER, rough endoplasmic reticulum.

Molecular evolutionary and genomic aspects of the bat fly endosymbionts

Nucleotide compositions of the 16S rRNA gene of the bat fly endosymbionts were adenine/thymine (AT) rich, ranging from 50.3% to 51.2%, in comparison with those of allied facultative *Arsenophonus* endosymbionts ranging from 45.8% to 47.0% (Figure 2). Evolutionary rates of the 16S rRNA, *gyrB* and *groEL* genes of the bat fly endosymbionts were

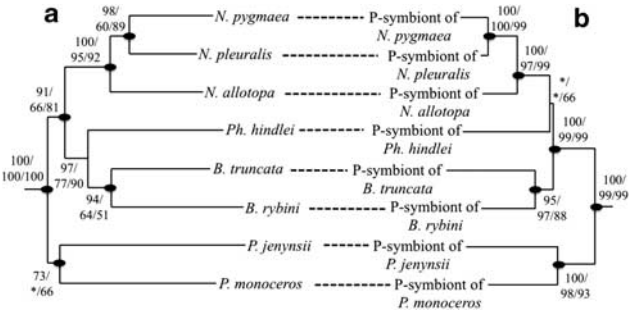

Figure 6 Comparison between the phylogeny of the nycteribiid bat fly species and the phylogeny of their endosymbionts. (**a**) A neighbor-joining phylogeny of the host insect species on the basis of the data set of Supplementary Figure S9. (**b**) A neighbor-joining phylogeny of their endosymbionts on the basis of the data set of Supplementary Figure S6. Dots on the nodes indicate co-divergence events inferred by the jungles algorithm (Charleston and Page, 2002). Bootstrap values are indicated as in Figure 2.

significantly higher than allied facultative *Arsenophonus* endosymbionts (Table 1). Pulsed-field gel electrophoresis of the bacteriomes of *P. jenynsii* estimated the genome size of the endosymbiont to be 0.76 Mb (Supplementary Figure S10).

Discussion

Host–symbiont co-evolution in nycteribiid bat flies

Among diverse insect endosymbionts of obligate nature, such as *Buchnera* of aphids (Moran *et al.*, 1993), *Wigglesworthia* of tsetse flies (Chen *et al.*, 1999), *Carsonella* of psyllids (Thao *et al.*, 2000), *Portiera* of whiteflies (Thao and Baumann, 2004), *Sulcia* of many homopterans (Moran *et al.*, 2005), *Baumannia* of sharpshooters (Takiya *et al.*, 2006), *Blochmannia* of carpenter ants (Sauer *et al.*, 2000), *Nardonella* of weevils (Conord *et al.*, 2008) and others, the endosymbiont phylogeny generally mirrors the host phylogeny, indicating stable and intimate host–symbiont association over evolutionary time. Our results unequivocally indicate that the endosymbiont of nycteribiid bat flies has experienced a similar co-evolutionary history (Figure 6). Vertical symbiont transmission through host generations should facilitate host–symbiont co-speciation. The presence of the endosymbiont cells in the milk glands (Figures 4e and f; Figure 5c) strongly suggests a uterine transmission route of the nycteribiid endosymbiont, wherein the endosymbiont is vertically passed from mother to offspring through milk gland secretion in the uterus, as has been reported from tsetse flies and other hippoboscoids (Buchner, 1965; Attardo *et al.*, 2008). The family Nycteribiidae consists of 3 subfamilies Nycteribiinae (7 genera, 210 species), Cyclopodinae (4 genera, 62 species) and Archinycteribiinae (1 genus, 3 species) (Dick and Patterson, 2006). All nycteribiid bat flies examined in this study (four genera, eight species)

Table 1 Relative rate test for comparing the molecular evolutionary rates of 16S rRNA, *gyrB* and *groEL* gene sequences between the lineage of nycteribiid endosymbionts and the lineage of facultative *Arsenophonus* endosymbionts

Gene (sites)[a]	*Lineage 1 (accession no.)*	*Lineage 2 (accession no.)*	*Outgroup (accession no.)*	*K1*[b]	*K2*[c]	*Difference of distance*[d]	*Rate ratio*[e]	P-*value*[f]
16S rRNA (1436)	Endosymbionts of *Penicillidia jenynsii* (AB632434) and *Nycteribia allotopa* (AB632414)	*Arsenophonus nasoniae* (AY264674) and *Arsenophonus insecticola* (DQ115536)	*Providencia stuartii* (AF008581)	0.049	0.022	0.027	2.2	1.6×10^{-4}
gyrB (642)	Endosymbionts of *P. jenynsii* (AB632475) and *N. allotopa* (AB632455)	*A. nasoniae* (FN545181)	*Escherichia coli* (NC_000913)	0.196	0.031	0.165	6.3	$< 1.0 \times 10^{-7}$
groEL (1054)	Endosymbionts of *P. jenynsii* (AB632516) and *N. allotopa* (AB632496)	*A. nasoniae* (FN545234)	*E. coli* (NC_000913)	0.062	0.027	0.035	2.3	5.8×10^{-4}

[a]Number of aligned nucleotide sites subjected to the analysis.
[b]Estimated mean distance between lineage 1 and the last common ancestor of lineages 1 and 2.
[c]Estimated mean distance between lineage 2 and the last common ancestor of lineages 1 and 2.
[d]K1–K2.
[e]K1/K2.
[f]*P*-value was generated using the program RRTree (Robinson-Rechavi and Huchon, 2000).

belong to the Nycteribiinae, suggesting that the origin of the host–symbiont association could be dated back to the common ancestor of the subfamily Nycteribiinae.

Reductive genome evolution of the nycteribiid endosymbiont

The AT-biased nucleotide composition (Figure 2), the accelerated molecular evolution (Table 1) and the reduced genome size (Supplementary Figure S10) consistently indicate that the nycteribiid endosymbiont has experienced a remarkable level of reductive genome evolution. The estimated genome size, 0.76 Mb, is similar to genome sizes of obligate insect endosymbionts like *Buchnera* of aphids (~0.6 Mb) (Shigenobu *et al.*, 2000) and *Wigglesworthia* of tsetse flies (~0.7 Mb) (Akman *et al.*, 2002), and remarkably smaller than the genome of allied facultative endosymbiont *Arsenophonus nasoniae* (~3.2 Mb) (Wilkes *et al.*, 2010), as well as the genomes of free-living gammaproteobacteria like *Escherichia coli* (~4.6 Mb) (Blattner *et al.*, 1997) and *Vibrio cholerae* (~4.0 Mb) (Heidelberg *et al.*, 2000). Such evolutionary patterns are probably attributable to the stable and nutrition-rich endocellular habitat for the endosymbiont and also attenuated purifying selection due to small population size and strong bottleneck associated with the lifestyle of the vertically transmitted endosymbiont (Wernegreen, 2002; Moran *et al.*, 2008).

Evolutionary origin of the nycteribiid endosymbiont

Molecular phylogenetic analyses revealed that the nycteribiid endosymbionts form a robust clade in the *Gammaproteobacteria*, and the clade is certainly distinct but allied to the clade of *Arsenophonus* spp., which embrace facultative endosymbionts of diverse insects (Figure 2). These patterns suggest that the evolutionary origin of the obligate nycteribiid endosymbiont might be an *Arsenophonus*-like facultative endosymbiont. In this context, it seems meaningful that facultative *Arsenophonus* endosymbionts have been detected from several *Nycteribia* species in addition to the obligate endosymbionts (Nováková *et al.*, 2009). The obligate bacteriome-associated endosymbionts of primate lice, *Riesia* spp., are also allied to the clade of *Arsenophonus* spp. (Sasaki-Fukatsu *et al.*, 2006; Allen *et al.*, 2007), which may represent a similar evolutionary trajectory from facultative to obligate endosymbiotic association. In the bedbug *Cimex lectularius*, its obligate bacteriome-associated endosymbiont was shown to have evolved from a *Wolbachia* lineage (Hosokawa *et al.*, 2010), the members of which are commonly found in diverse insects as facultative and/or parasitic endosymbiotic associates (Bourtzis and Miller, 2003; Werren *et al.*, 2008).

Commonality and variability of the endosymbiotic system among nycteribiid bat flies

A previous study (Aschner, 1931) identified loose clusters of bacteriocytes in the abdominal body cavity of bat flies of the genus *Nycteribia*, which was confirmed by our histological observation of *N. allotopa* (Supplementary Figure S2). In *P. jenynsii*, by contrast, we discovered a previously unknown type of symbiotic organ: in adult females, many bacteriocytes constitute a pair of distinct bacteriomes inside the swellings of the fifth abdominal ventral plate (Figure 4). These observations suggest that different symbiotic organs have evolved in the subfamily Nycteribinae, whereas the

endosymbionts harbored therein have been conserved. Meanwhile, endosymbiont localization in the milk glands has been consistently observed among all nycteribiid bat flies ever examined histologically: *Nycteribia* spp., *P. jenynsii* and *E. aegyptia* (Aschner, 1931, 1946; Figure 4), which should be relevant to the importance of uterine vertical transmission of the endosymbiont for the host bat flies.

Relationship to endosymbionts of other hippoboicoid families

The families Glossinidae (tsetse flies), Hippoboscidae (louse flies), Streblidae (sterblid bat flies) and Nycteribiidae (nycteribiid bat flies) form a well-defined superfamily Hippoboscoidea (McAlpine, 1989; Nirmala *et al.*, 2001; Petersen *et al.*, 2007), all members of which are obligate blood feeders and commonly associated with bacterial endosymbionts (Roubaud, 1919; Zacharias, 1928; Aschner, 1931; Buchner, 1965), although the endosymbionts are microbiologically distinct between the host families (see Figure 2). Tsetse flies are associated with *W. glossinidia*, which is phylogenetically not related to *Arsenophonus* spp. in the *Gammaproteobacteria* (Aksoy, 1995). Meanwhile, diverse *Arsenophonus*-related endosymbionts have been detected from louse flies and stereblid bat flies (Dale *et al.*, 2006; Trowbridge *et al.*, 2006; Nováková *et al.*, 2009), which are certainly allied to but distinct from the clade of nycteribiid endosymbionts (Figure 2; Nováková *et al.*, 2009). These patterns favor the hypothesis that the hippoboscoid endosymbionts are of multiple evolutionary origins and have experienced repeated acquisitions and/or replacements from different bacterial sources to meet their physiological requirement for their obligate blood-feeding lifestyle.

Biological function of the endosymbiont for nycteribiid bat flies

On account of the long-lasting host–symbiont association in the Nycteribiidae (Figure 6), it seems plausible that the endosymbiont has important biological roles for its nycteribiid host, although its exact function is yet to be established. As for other endosymbionts of obligate blood-feeding insects such as *Wigglesworthia* of tsetse fly, *Riesia* of human body louse and *Wolbachia* of bedbug, their biological function has been demonstrated as provisioning of B vitamins that are deficient in vertebrate blood (Puchta, 1955; Nogge, 1981; Hosokawa *et al.*, 2010). It is conceivable that the nycteribiid endosymbiont has a similar role for the host insect. Endosymbiont genome analyses revealed that biosynthetic genes for B vitamins are encoded in the main chromosome of *Wigglesworthia* (Akman *et al.*, 2002) while present in a plasmid of *Riesia* (Kirkness *et al.*, 2010). Sequencing of the genome of the nycteribiid endosymbiont will provide a crucial insight into its biological function, in particular involvement in provisioning of B vitamins.

Proposal of candidate name

On account of those distinct and coherent microbiological, phylogenetic and evolutionary traits described above, we propose the designation '*Candidatus* Aschnera chinzeii' for the endosymbiotic bacterial clade associated with bat flies of the family Nycteribiidae. The generic name honors Manfred Aschner, who first described the endosymbionts of nycteribiid bat flies (Aschner, 1931, 1946). The specific name is after a Japanese biologist Yasuo Chinzei, who significantly contributed to the biochemistry and molecular biology of blood-sucking insects (Yuda *et al.*, 1996; Kadota *et al.*, 2004).

Acknowledgements

We thank Hajime Hashimoto, Kuniko Kawai, Akio Kobayashi, Norihisa Kondo, Ken-ichiro Maruyama, Kazue Okumura, Naomi Seki, Nobutaka Urano, Noboru Yaguchi and Hajime Yoshino for insect samples, and Yoichi Kamagata for logistic supports. This study was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN).

References

Adams TS. (1999). Hematophagy and hormone release. *Ann Entomol Soc Am* **92**: 1–13.

Allen JM, Reed DL, Perotti MA, Braig HR. (2007). Evolutionary relationships of '*Candidatus* Riesia spp.,' endosymbiotic enterobacteriaceae living within hematophagous primate lice. *Appl Environ Microbiol* **75**: 1659–1664.

Akman L, Yamashita A, Watanabe H, Oshima K, Shiba T, Hattori M *et al.* (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nat Genet* **32**: 402–407.

Aksoy S. (1995). *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. *Int J Syst Bacteriol* **45**: 848–851.

Aksoy S. (2000). Tsetse-a haven for microorganisms. *Parasitol Today* **16**: 114–118.

Aksoy S, Rio RVM. (2005). Interactions among multiple genomes: Tsetse, its symbionts and trypanosomes. *Insect Biochem Mol Biol* **35**: 691–698.

Aschner M. (1931). Die Bakterienflora der Pupiparen (Diptera). Eine Symbiosestudie an blutsaugenden Insekten. *Z Morphol Ökol Tiere* **20**: 368–442.

Aschner M. (1946). The symbiosis of *Eucampsipoda aegyptia* Mcq. (Diptera, Pupipara: Nycteribiidae). *Bull Soc Fouad I Entomol* **30**: 1–6.

Attardo GM, Lohs C, Heddi A, Alam UH, Yildirim S, Aksoy S. (2008). Analysis of milk gland structure and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity. *J Insect Physiol* **54**: 1236–1242.

Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M *et al.* (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1474.

Bourtzis K, Miller TA. (2003). *Insect Symbiosis*. CRC Press: Boca Raton, FL.

Breznak JA, Brune A. (1994). Role of microorganisms in the digestion of lignocellulose by termites. *Annu Rev Entomol* **39**: 453–487.

Buchner P. (1965). *Endosymbiosis of Animals with Plant Microorganisms*. Interscience: New York.

Charleston MA, Page RDM. (2002) TreeMap v2.0. Application for Apple Macintosh (http://sydney.edu.au/engineering/it/~mcharles/software/treemap/treemap.html).

Chen X, Li S, Aksoy S. (1999). Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. *J Mol Evol* **48**: 49–58.

Conord C, Despres L, Vallier A, Balmand S, Miquel C, Zundel S *et al.* (2008). Long-term evolutionary stability of bacterial endosymbiosis in Curculionoidea: additional evidence of symbiont replacement in the Dryophthoridae family. *Mol Biol Evol* **25**: 859–868.

Dale C, Maudlin I. (1999). *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. *Int J Syst Bacteriol* **49**: 267–275.

Dale C, Beeton M, Harbison C, Jones T, Pontes M. (2006). Isolation, pure clture, and characterization of '*Candidatus* Arsenophonus arthropodicus,' an intracellular secondary endosymbiont from the hippoboscid louse fly *Pseudolynchia canariensis*. *Appl Environ Microbiol* **72**: 2997–3004.

Dick CW, Patterson BD. (2006). Bat flies: obligate ectoparasites of bats. In: Morand S, Krasnov BR, Poulin R (eds), *Micromammals and Macroparasites: From Evolutionary Ecology to Management*. Springer: Tokyo, pp 179–194.

Douglas AE. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu Rev Entomol* **43**: 17–37.

Fukatsu T. (1999). Acetone preservation: a practical technique for molecular analysis. *Mol Ecol* **8**: 1935–1945.

Fukatsu T, Nikoh N, Kawai R, Koga R. (2000). The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl Environ Microbiol* **66**: 2748–2758.

Grimardi D, Engel MS. (2005). *Evolution of the Insects*. Cambridge University Press: Cambridge.

Guindon S, Gascuel O. (2003). A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**: 696–704.

Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ *et al.* (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholelae*. *Nature* **406**: 477–483.

Hosokawa T, Kikuchi Y, Nikoh N, Shimada M, Fukatsu T. (2006). Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. *PLoS Biol* **4**: e337.

Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T. (2010). *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc Natl Acad Sci USA* **107**: 769–774.

Kadota K, Ishino T, Matsuyama T, Chinzei Y, Yuda M. (2004). Essential role of membrane-attack protein in malarial transmission to mosquito host. *Proc Natl Acad Sci USA* **101**: 16310–16315.

Kikuchi Y, Hosokawa T, Nikoh N, Meng XY, Kamagata Y, Fukatsu T. (2009). Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs. *BMC Biol* **7**: 2.

Kirkness EF, Haas BJ, Sun W, Braig HR, Perotti MA, Clark JM *et al.* (2010). Genome sequences of the human body louse and its primary endosymbiont provide insights into the permanent parasitic lifestyle. *Proc Natl Acad Sci USA* **107**: 12168–12173.

Koga R, Tsuchida T, Fukatsu T. (2009). Quenching autofluorescence of insect tissues for *in situ* detection of endosymbionts. *Appl Entomol Zool* **44**: 281–291.

Lehane M. (2005). *The Biology of Blood-Sucking in Insects*. Cambridge University Press: Cambridge.

McAlpine JF. (1989). Phylogeny and classification of the Muscomorpha. In: McAlpine JF (ed), *Manual of Nearctic Diptera, vol. 3, Monograph 32*. Agriculture Canada: Ottawa, pp 1397–1518.

Moran MA, Munson MA, Baumann P, Ishikawa H. (1993). A molecular clock in endosymbiotic bacteria is calibrated using the insect host. *Proc R Soc Lond B* **253**: 167–171.

Moran NA, Tran P, Gerardo NM. (2005). Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum *Bacteroidetes*. *Appl Environ Microbiol* **1**: 8802–8810.

Moran NA, McCutcheon JP, Nakabachi A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet* **42**: 165–190.

Nirmala X, Hypša V, urovec M. (2001). Molecular phylogeny of Calyptratae (Diptera: Brachycera): the evolution of 18S and 16S ribosomal rDNAs in higher dipterans and their use in phylogenetic inference. *Insect Mol Biol* **10**: 475–485.

Nogge G. (1981). Significance of symbionts for the maintenance of an optimal nutritional state for successful reproduction in haematophagous arthropods. *Parasitology* **82**: 101–104.

Nováková E, Hypsa V, Moran NA. (2009). *Arsenophonus*, an emerging clade of intracellular symbionts with a broad host distribution. *BMC Microbiol* **9**: 143.

Ohkuma M. (2003). Termite symbiotic systems: efficient bio-recycling of lignocellulose. *Appl Microbiol Biotechnol* **61**: 1–9.

Petersen FT, Meier R, Kutty SN, Wiegmann BM. (2007). The phylogeny and evolution of host choice in the Hippoboscoidea (Diptera) as reconstructed using four molecular markers. *Mol Phylogenet Evol* **45**: 111–122.

Puchta O. (1955). Experimentelle Untersuchungen über die Bedeutung der Symbiose der Kleiderlaus *Pediculus vestimenti* Burm. *Z Parasitenk* **17**: 1–40.

Robinson-Rechavi M, Huchon D. (2000). RRTree: relative-rate tests between groups of sequences on a phylogenetic tree. *Bioinformatics* **16**: 296–297.

Roubaud B. (1919). Les particularites de la nutrition et de la vie symbiotique chez les mouches tsetse. *Ann Inst Pasteur* **33**: 489–537.

Sasaki-Fukatsu K, Koga R, Nikoh N, Yoshizawa K, Kasai S, Mihara M *et al.* (2006). Symbiotic bacteria associated with stomach discs of human lice. *Appl Environ Microbiol* **72**: 7349–7352.

Sauer C, Stackebrandt E, Gadau J, Holldobler B, Gross R. (2000). Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus*

Blochmannia gen. nov. *Int J Syst Evol Microbiol* **50**: 1877–1886.
Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407**: 81–86.
Swofford DL. (2001). PAUP* Version 4.0b10 [computer program]. In: 4.0b10 edition. Sinauer: Sunderland, MA.
Takiya DM, Tran PL, Dietrich CH, Moran NA. (2006). Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. *Mol Ecol* **15**: 4175–4191.
Thao ML, Baumann P. (2004). Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Appl Environ Microbiol* **70**: 3401–3406.
Thao ML, Moran NA, Abbot P, Brennan EB, Burckhardt DH, Baumann P. (2000). Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Appl Environ Microbiol* **66**: 2898–2905.
Thompson JD, Higgins DG, Gibson TJ. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.
Toh H, Weiss BL, Perkin SAH, Yamashita A, Oshima K, Hattori M *et al.* (2006). Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. *Genome Res* **16**: 149–156.
Trowbridge RE, Dittmar K, Whiting MF. (2006). Identification and phylogenetic analysis of *Arsenophonus*- and *Photorhabdus*-type bacteria from adult Hippoboscidae and Streblidae (Hippoboscoidea). *J Invertebr Pathol* **91**: 64–68.
Wernegreen JJ. (2002). Genome evolution in bacterial endosymbionts of insects. *Nat Rev Genet* **3**: 850–861.
Werren JH, Baldo L, Clark ME. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* **6**: 741–751.
Wilkes TE, Darby AC, Choi JH, Colbourne JK, Werren JH, Hurst GDD. (2010). The draft genome sequence of *Arsenophonus nasoniae*, son-killer bacterium of *Nasonia vitripennis*, reveals genes associated with virulence and symbiosis. *Insect Mol Biol* **19**(s1): 59–73.
Yuda M, Hirai M, Miura K, Matsumura H, Ando K, Chinzei Y. (1996). cDNA cloning, expression and characterization of nitric-oxide synthase from the salivary glands of the blood-sucking insect *Rhodnius prolixus*. *Eur J Biochem* **242**: 807–812.
Zacharias A. (1928). Untersuchungen über die intracellulare Symbiose bei den Pupiparen. *Z Morphol Ökol Tiere* **10**: 676–737.

Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)