

# Phylogenetic Position and Peculiar Genetic Traits of a Midgut Bacterial Symbiont of the Stinkbug *Parastrachia japonensis*<sup>†</sup>

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**The stinkbug *Parastrachia japonensis* (Hemiptera: Parastrachiidae) is known for its prolonged prereproductive nonfeeding period, maternal care of eggs in an underground nest, and maternal collection and provisioning of food (fruits) for nymphs. A previous study suggested that a bacterial symbiont is involved in uric acid recycling in this insect during the nonfeeding period, but the identity of this symbiont has not been determined. Here we characterized a novel bacterial symbiont obtained from *P. japonensis*. Molecular phylogenetic analyses based on 16S rRNA, *gyrB*, and *groEL* gene sequences consistently indicated that this symbiont constituted a distinct lineage in the *Gammaproteobacteria* that has no close relatives but is allied with gut symbionts of acanthosomatid and plataspid stinkbugs, as well as with endocellular symbionts of sharpshooters, tsetse flies, and aphids. The symbiont genes had a remarkably AT-biased nucleotide composition and exhibited significantly accelerated molecular evolution. The symbiont genome was extremely reduced; its size was estimated to be 0.85 Mb. These results suggest that there has been an intimate host-symbiont association over evolutionary time. The symbiont was localized in swollen crypts in a posterior part of the midgut, which was a specialized symbiotic organ. The possibility that the symbiont is involved in uric acid recycling is discussed. The designation “*Candidatus Benitsuchiphilus tojo*” is proposed for the symbiont.**

Many insects have mutualistic associations with symbiotic microorganisms inhabiting the gut, body cavity, or cells, in which the symbionts play important roles in the survival and reproduction of their hosts, including food digestion and nutrient provisioning (4, 5). Most, but not all, heteropteran plant-sucking stinkbugs of the infraorder Pentatomomorpha harbor bacterial symbionts in the midgut (5, 10). It has been reported that in some stinkbug species of the families Pentatomidae, Plataspidae, Acanthosomatidae, Cydnidae, and Alydidae, nymphs exhibit retarded growth, reduced body size, and/or high mortality when they are experimentally deprived of their symbionts (1, 5, 8, 16, 22, 23, 27, 30, 37).

Parastrachiidae is a small stinkbug family containing only one genus and two species, *Parastrachia japonensis* and *Parastrachia nagaensis* (36, 39), although recent comprehensive phylogenetic analyses suggested that the genera *Parastrachia* and *Dismegistus* form a monophyletic group (11). *P. japonensis* (Fig. 1A) is known for its peculiar ecology and behavior, including a prolonged prereproductive nonfeeding period for newly emerged adults that lasts for 9 months, maternal care of eggs in an underground nest, and maternal collection and provisioning of food (fruits) for nymphs (7, 41, 43). A previous study reported that when adult insects were treated with an antibiotic in the nonfeeding period, the uricase activity in the

midgut was significantly reduced and the mortality of the insects increased dramatically (20). Thus, it was suggested that a bacterial symbiont is involved in uric acid recycling in this host insect during the nonfeeding period, like the bacterial symbionts of termites (28) and cockroaches (6, 34) and a yeast-like symbiont of rice planthoppers (35).

Recent microbiological studies have revealed a diverse array of stinkbug symbionts and their lifestyles, including vertically transmitted gammaproteobacterial symbionts in pentatomid, plataspid, acanthosomatid, and scutellerid stinkbugs (8, 16, 18, 23, 29, 30, 31), environmentally acquired betaproteobacterial symbionts (*Burkholderia* spp.) in alydid stinkbugs (21, 22), and vertically and horizontally transmitted actinobacterial symbionts in pyrrhocorid stinkbugs (19). For the parastrachiid symbiont, the only previous study was performed mainly to examine insect physiology (20), and there was no microbiological characterization of the symbiont. In this study, therefore, we attempted to characterize the bacterial symbiont of *P. japonensis*.

## MATERIALS AND METHODS

**Insects.** *P. japonensis* adults were collected at seven localities in Japan from 2007 to 2009 (see Table S1 in the supplemental material). The collected insects were kept in plastic cases with moistened paper towels and used for experiments within several days.

**Insect dissection and DNA extraction.** Adult insects were dissected in sterile phosphate-buffered saline (PBS) (0.8% NaCl, 0.02% KCl, 0.115% Na<sub>2</sub>HPO<sub>4</sub>, 0.02% KH<sub>2</sub>PO<sub>4</sub> [pH 7.5]) using fine forceps and microscissors. The alimentary tract (Fig. 1B) was isolated and subjected to either histological analysis or DNA extraction using a NucleoSpin tissue kit (Macherey-Nagel).

**Cloning and sequencing.** Three bacterial genes, the 16S rRNA, *gyrB*, and *groEL* genes, were cloned and sequenced using DNA samples from the midgut fourth section with crypts. PCR amplification was conducted using AmpliTaq DNA polymerase (Applied Biosystems) and primers 16SA1 (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16SB1 (5'-TAC GGY TAC CTT GTT ACG ACT

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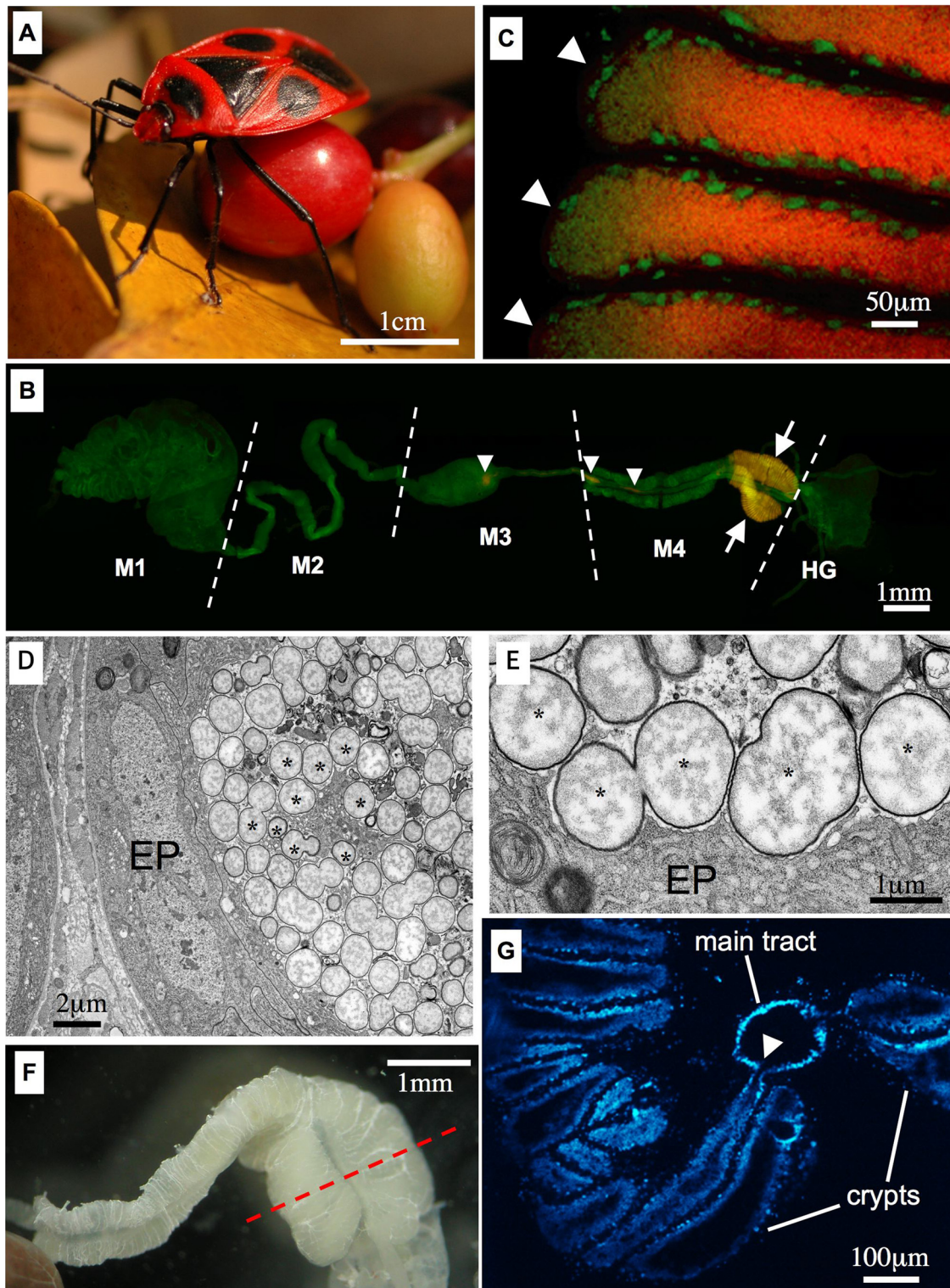


FIG. 1. (A) Adult female of *P. japonensis* carrying fruit. (B and C) *In situ* hybridization targeting 16S rRNA of the symbiont. Red and green indicate the symbiont and the host nucleus, respectively; yellow is due to superimposed red and green signals. (B) Dissected female alimentary tract. The arrows indicate concentrated symbiont signals in the specialized symbiotic organ, swollen midgut crypts, while the arrowheads indicate sporadic symbiont signals in the midgut main tract. Abbreviations: M1, midgut first section; M2, midgut second section; M3, midgut third section; M4, midgut fourth section with crypts; HG, hindgut. (C) Enlarged image of the swollen midgut crypts. There are strong symbiont signals in the lumen of the crypts. Each arrowhead indicates a crypt. (D and E) Transmission electron microscopy of the swollen midgut crypt. Asterisks indicate symbiont cells. EP, epithelial cells of the crypt. (D) Symbiont cells restricted to the lumen of a crypt. (E) Enlarged image of symbiont cells. (F) Dissected midgut fourth section from an adult female. (G) Section of the midgut fourth section approximately through the plane indicated by the dashed line in panel F. The arrowhead indicates the connection between a crypt and the main tract of the midgut.

T-3') for the 16S rRNA gene (9), primers gyrBsymF (5'-TTA TCA TGA CWG TAT TAC ATG CWG G-3') and gyrBsymR (5'-TCC AGC WGA ATC WCC TTC WAC-3') for the *gyrB* gene, and primers groELIF (5'-ATG GCA GCW AAA GAC GTA AAT TYG G-3') and groELIR (5'-TTA CAT CAT KCC GCC CAT GC-3') for the *groEL* gene (17). The PCR products (1.5 kb for the 16S rRNA gene, 1.0 kb for the *gyrB* gene, and 1.6 kb for the *groEL* gene) were cloned and sequenced as described previously (21).

**Molecular phylogenetic and evolutionary analyses.** Multiple alignments of the nucleotide sequences were generated using the program Clustal W (42). The alignments were then inspected and corrected manually, and ambiguously aligned sites were removed. Phylogenetic analyses were conducted by using three methods, the maximum parsimony (MP), maximum likelihood (ML), and Bayesian (BA) methods, using the programs PAUP 4.0b10 (40), Phylml (12), and MrBayes v3.0b4 (33), respectively. In the MP analysis, all sites and character changes were weighted equally. In the ML and BA analyses, the GTR+I+G model was selected for the 16S rRNA, *gyrB*, and *groEL* gene phylogenies on the basis of the Akaike criterion using the program MrModeltest v2.1 (<http://www.ebc.uu.se/systzoo/staff/nylander.html>). Bootstrap tests were performed with 100 replications in the MP and ML analyses. In the BA analysis, a total of 11,000 trees were obtained for the 16S rRNA gene (ngen = 160,000, samplefreq = 10, burn in = 5,000), and 8,000 trees were obtained for the *gyrB* and *groEL* genes (ngen = 100,000, samplefreq = 10, burn in = 2,000). Based on the data, a 50% majority rule consensus tree was constructed. Relative rate tests were performed using the program RRTree (32) based on genetic distances estimated by using Kimura's two-parameter model (24).

**Histology.** Alimentary tracts dissected from female insects were fixed in Carnoy's solution (ethanol-chloroform-acetic acid, 6:3:1) overnight, treated with 6% hydrogen peroxide in 80% ethanol for 3 days to eliminate autofluorescence (25), and then kept in 100% ethanol at room temperature. For tissue sectioning, the preserved samples were cleared using an ethanol-xylene series, embedded in paraffin, and cut into 5- $\mu$ m serial sections. The tissue sections were mounted on silane-coated glass slides, dewaxed using a xylene-ethanol series, and air dried prior to *in situ* hybridization. For whole-mount *in situ* hybridization, the preserved samples were washed twice in PBSTx (PBS containing 0.3% Triton X-100) and directly subjected to the hybridization procedure.

**In situ hybridization.** A fluorochrome-labeled oligonucleotide probe, BNTC129-A555 (5'-Alexa555-CCT CTA ATT AGG CAG ATC C-3') targeting 16S rRNA of the symbiont, was used. Tissue samples were incubated with a hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% SDS, 30% formamide) containing 50 nM probe. For counterstaining of host insect nuclei, 4  $\mu$ M 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen) or 0.5  $\mu$ M SYTOX green (Invitrogen) was added to the hybridization buffer. After overnight incubation, the samples were thoroughly washed in PBSTx and mounted in Slowfade antifade solution (Molecular Probes). The sectioned and whole-mount samples were observed with an epifluorescence microscope (Axiophot; Carl Zeiss) and/or a laser scanning confocal microscope (LSCM Pascal5; Carl Zeiss). To confirm the specificity of detection, a series of control experiments were conducted as described previously (23).

**Electron microscopy.** The midgut fourth section was dissected from adult females in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The tissue was prefixed with the fixative at 4°C for 3 h and postfixed in 2% osmium tetroxide for 60 min at 4°C. After dehydration using an ethanol series, the materials were embedded in Spurr resin and cut into ultrathin sections (thickness, 75 nm), which were double stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (H-7000; Hitachi).

**Pulsed-field gel electrophoresis.** The dissected midgut fourth sections were homogenized in phosphate-buffered saline containing 10 mM EDTA and filtered through a 20- $\mu$ m nylon net filter (Millipore). The symbiont cells obtained were embedded in 1% low-melting-point agarose, and agarose plugs were treated with proteinase K at 50°C overnight. After thorough washing, the symbiont DNA trapped in a plug was digested with a restriction enzyme (I-CeuI) and subjected to pulsed-field gel electrophoresis using a CHEF Mapper XA (Bio-Rad).

**Nucleotide sequence accession numbers.** The nucleotide sequences reported in this study have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB548048 to AB548068.

## RESULTS AND DISCUSSION

**Phylogenetic placement of the symbiont of *P. japonensis*.** The 16S rRNA gene sequences were identical within and between the local populations of *P. japonensis*. Phylogenetic analyses showed that the symbiont of *P. japonensis* formed a distinct

lineage in the *Gammaproteobacteria*, which had no close relatives but was allied with gut symbionts of acanthosomatid and plataspid stinkbugs, as well as with endocellular symbionts of sharpshooters, tsetse flies, and aphids (Fig. 2). Similar results were obtained with the *gyrB* and *groEL* gene sequences; the symbiont sequences were identical within and between the local populations and constituted a distinct lineage in the *Gammaproteobacteria* (see Fig. S1 and S2 in the supplemental material).

### Reductive genome evolution in the symbiont of *P. japonensis*.

The three symbiont gene sequences consistently had a remarkably AT-biased nucleotide composition (Fig. 2; see Fig. S1 and S2 in the supplemental material) and exhibited significantly accelerated molecular evolution compared with free-living gammaproteobacteria (Table 1). The size of the symbiont genome was estimated to be 0.85 Mb (Fig. 3), which was similar to the sizes of the genomes of obligate endocellular symbionts like *Buchnera* in aphids (~0.6 Mb) (38) and *Wigglesworthia* in tsetse flies (~0.7 Mb) (2), as well as the genomes of obligate gut symbionts like *Ishikawaella* in plataspid stinkbugs (~0.8 Mb) (16) and *Rosenkranzia* in acanthosomatid stinkbugs (~0.9 Mb) (23), but the symbiont genome was remarkably smaller than the genomes of free-living gammaproteobacteria like *Escherichia coli* (~4.6 Mb) (3) and *Vibrio cholerae* (~4.0 Mb) (13). This peculiar genetic trait has been identified in ancient endocellular symbiotic bacteria that cospeciated with their hosts (2, 38) and also in some gut symbiotic bacteria of stinkbugs (16, 23). The universal trend toward reductive genome evolution in the obligate mutualistic symbionts has been hypothesized to be related to attenuated purifying selection due to a small population size and substantial bottleneck, which are associated with the lifestyle of vertically transmitted symbionts (16, 26, 44). In this context, it is conjectured that the symbiont of *P. japonensis* has similarly been subjected to strict vertical transmission from mother to offspring over evolutionary time, presumably establishing an obligate mutualistic association. Recent comprehensive phylogenetic analyses suggested that stinkbugs of the genera *Parastrachia* and *Dismegistus* form a monophyletic group (11). Biological and coevolutionary aspects of the host-symbiont association in these stinkbug groups should be investigated.

**In vivo localization of the symbiont in *P. japonensis*.** *In situ* hybridization targeting 16S rRNA of the symbiont identified strong signals in the swollen crypts in the posterior part of the midgut fourth section (Fig. 1B and C). In contrast, few symbiont signals were observed in the smaller crypts in the anterior part of the midgut fourth section (Fig. 1B). In the other parts of the alimentary tract there were few symbiont signals, except for sporadic signals occasionally seen in the main tract of the midgut (Fig. 1B). Transmission electron microscopy showed that the lumen of the swollen crypts was filled with globular bacterial cells with a uniform morphotype (Fig. 1D and E), whereas the lumen of the smaller crypts in the anterior part was populated by few bacteria (data not shown). In the midgut fourth section, many crypts were arranged in two rows and fused into a ribbon-shaped structure (Fig. 1F). Histological examination confirmed that the lumen of the crypts was connected to the lumen of the midgut main tract (Fig. 1G). These results indicate that in *P. japonensis* (i) the midgut fourth section is differentiated into a swollen posterior part and a



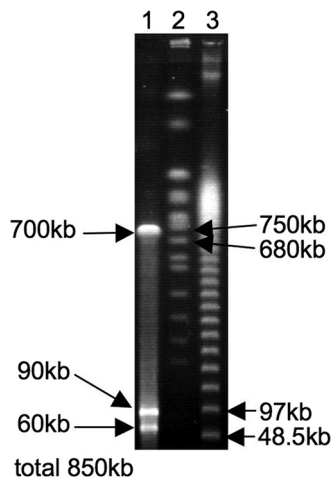


FIG. 3. Genome size of the symbiont of *P. japonensis* estimated by pulsed-field gel electrophoresis. Lane 1, symbiont genomic DNA digested with I-CeuI; lanes 2 and 3, DNA size markers.

(14), suggesting that the trophic eggs may contribute not only nutrients but also the beneficial symbiont to the offspring, as the mother-made symbiont capsules do in the Plataspidae (8, 15, 16). Females of *P. japonensis* lay an egg mass in the nest, remain near the eggs until they hatch, and collect and provision fruits of *Schoepfia jasminodora* for the nymphs (43). Hence, it is conceivable that the symbiont is passed directly from the mother to nymphs during maternal care, as reported previously for a cydnid species (37).

**Biological role of the symbiont in *P. japonensis*.** A previous study suggested that a bacterial symbiont is involved in uric acid recycling in the prereproductive nonfeeding period in *P. japonensis* (20). Considering the highly developed symbiotic system (Fig. 1) and the presumably long-lasting host-symbiont association (Fig. 2 and 3 and Table 1), it appears likely that the gut symbiont characterized in this study plays a biological role in uric acid recycling. Of course, the possibility that other minor bacterial associates that we failed to detect in this study are involved in uric acid recycling cannot be ruled out. Whether the symbiont itself has uricolytic activity or the uricolysis of the host insect is affected by symbiont infection should be examined in future studies.

**Proposal of candidate name.** On the basis of the results described above, we propose the name “*Candidatus* Benitsuchiophilus toji” for the novel symbiont lineage. The generic name indicates the association with *P. japonensis* and refers to the Japanese name of this insect (Benitsuchi-Kamemushi). The specific epithet refers to Sumio Tojo, who, along with colleagues, proposed that there is symbiont-mediated uricolytic activity in *P. japonensis* during the prereproductive nonfeeding period (20).

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