Infection prevalence of *Sodalis* symbionts among stinkbugs

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**Abstract**

**Introduction:** Diverse insects and other organisms are associated with microbial symbionts, which often significantly contribute to growth and survival of their hosts and/or drastically affect phenotypes of their hosts in a variety of ways. *Sodalis glossinidius* was first identified as a facultative bacterial symbiont of tsetse flies, and recent studies revealed that *Sodalis*-allied bacteria encompass diverse ecological niches ranging from free-living bacteria through facultative symbionts to obligate symbionts associated with a diverse array of insects. Despite potential ecological and evolutionary relevance of the *Sodalis* symbionts, their infection prevalence in natural insect populations has been poorly investigated.

**Results:** Here we surveyed diverse stinkbugs and allied terrestrial heteropteran bugs, which represented 17 families, 77 genera, 108 species, 310 populations and 960 individuals, for infection with the *Sodalis* symbionts. Diagnostic PCR detected relatively low infection frequencies of the *Sodalis* symbionts: 13.6% (14/103) of the species, 7.5% (22/295) of the populations, and 4.3% (35/822) of the individuals of the stinkbugs except for those belonging to the family Urostylididae. Among the urostylidid stinkbugs, strikingly, the *Sodalis* symbionts exhibited very high infection frequencies: 100% (5/5) of the species, 100% (15/15) of the populations, and 94.2% (130/138) of the individuals we examined. Molecular phylogenetic analysis based on bacterial 16S rRNA gene sequences revealed that all the symbionts were placed in the clade of *Sodalis*-allied bacteria while the symbiont phylogeny did not reflect the systematics of their stinkbug hosts. Notably, the *Sodalis* symbionts of the urostylidid stinkbugs were not clustered with the *Sodalis* symbionts of the other stinkbug groups on the phylogeny, suggesting their distinct evolutionary trajectories.

**Conclusions:** The relatively low infection frequency and the overall host-symbiont phylogenetic incongruence suggest that the *Sodalis* symbionts are, in general, facultative symbiotic associates in the majority of the stinkbug groups. On the other hand, it is conceivable, although speculative, that the *Sodalis* symbionts may play some substantial biological roles for their host stinkbugs of the Urostylididae.

**Keywords:** *Sodalis*, Hemiptera, Heteroptera, Stinkbug, Facultative symbiont, Infection frequency, Natural population

**Introduction**

Diverse insects are associated with symbiotic microorganisms [1]. Some symbionts are obligate companions essential for their hosts via, for example, provisioning of essential nutrients deficient in their host's diets, and often referred to as the primary symbionts [2,3]. Other symbionts are facultative associates not essential for their hosts, and often designated as the secondary symbionts [4,5]. Although not needed for their host's survival, many of the facultative symbionts drastically affect various adaptive phenotypes of their hosts, which include manipulating host's reproductive phenotypes in selfish ways [6,7], conferring host's resistance to parasites and pathogens [8-10], enhancing host's tolerance to heat stress [11,12], broadening host's food plant range [13,14], modifying host's body color [15,16] and others.

Grasping infection prevalence of these symbionts is important for gaining insights into biological interactions with their hosts. The primary symbionts of obligate nature generally exhibit 100% infection frequencies in
their host populations due to their indispensable roles. By contrast, the secondary symbionts of facultative nature exhibit variable infection frequencies ranging from near 0% to almost 100% depending on the symbiont species, the host species and populations, the environmental conditions, etc. For example, some Wolbachia strains attain 100% infection frequencies in their host populations by their selfish driving mechanisms such as cytoplasmic incompatibility and parthenogenesis induction [6,7]. The facultative symbionts Serratia, Regiella and Hamiltonella in natural aphid populations exhibit intermediate values between 0% to 100% [17-20], which probably reflect their context-dependent fitness consequences [8,9,11,13].

*Sodalis glossoeidius* was first identified and described as a gammaproteobacterial secondary symbiont of tsetse flies (Diptera: Glossinidae) [21-23]. Subsequently, primary symbionts associated with bacteriocytes of *Sitophilus* grain weevils (Coleoptera: Curculionidae) turned out to be closely related to *Sodalis* [24,25] and designated as ‘Candidatus Sodalis pierantoni’ [26]. Recently, a number of studies have reported occurrences of *Sodalis*-allied bacteria in a diverse array of insects: as bacteriocyte-associated presumable primary symbionts in bird lice (Phthiraptera: Philopteridae) [27,28], louse flies (Diptera: Hippoboscidae) [29,30], spittle bugs (Hemiptera: Cercopidea) [31,32] and pseudococcids (Hemiptera: Pseudococcidae) [33]; and as presumable secondary symbionts in acorn weevils (Coleoptera: Curculionidae) [34-36], longicorn beetles (Coleoptera: Cerambycidae) [37], stinkbugs (Hemiptera: Scutelleridae and Pentatomidae) [38-41], psyllids (Hemiptera: Triozidae) [42] and archaeococcoid scale insects (Hemiptera: Coccopterae) [43]. Furthermore, a *Sodalis*-allied bacterial strain was isolated from a human wound infection [44], and a biofilm-forming bacterium isolated from a tufa deposit, *Biostraticola tofii*, turned out to be a close relative of *Sodalis* [45], uncovering diverse ecological niches and symbiotic statuses of the *Sodalis*-allied bacteria. While infection frequencies in natural insect populations have been extensively surveyed for *Wolbachia* [46-48], *Rickettsia* [49,50], *Cardinium* [51-53], *Spiroplasma* [52,54], *Arsenophonus* [52,55] and other facultative symbionts, no systematic and comprehensive survey of *Sodalis* symbionts in natural host populations has been reported.

In this study, we surveyed diverse stinkbugs and allied terrestrial heteropteran bugs (order Hemiptera: suborder Heteroptera:infraorder Pentatomomorpha), which represent 17 families, 77 genera, 108 species, 310 populations and 960 individuals, for infection with *Sodalis* symbionts by diagnostic PCR and molecular phylogenetic approaches.

Materials and methods

Insect samples
Additional file 1 lists the insect samples examined in this study. These insects were preserved in either acetone or ethanol [56], or freshly brought to the laboratory. For large specimens, dissected gonad was subjected to DNA extraction. For small specimens, dissected abdomen was subjected to DNA extraction. DNA extraction was performed using QIAamp DNA Mini kit (Qiagen).

PCR, cloning and sequencing
A 1.5 kb region of the bacterial 16S rRNA gene was amplified by PCR with primers 16SA1 (5′-AGA TTT GTC TCM TGG CTC AG-3′) and 16SB1 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′), and cloned and sequenced as described previously [57]. Diagnostic PCR was performed with primers sodalis370F (5′-CGR TRG CGT TAA YAG CGC-3′) [38] and 16SB1 under the temperature profile of 95°C for 10 min followed by 35 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 1.5 min. For quality control of the DNA samples, a 1.5 kb region of mitochondrial 16S rRNA gene was amplified by PCR with primers Mtra1 (5′-AAW AAA CTA GGA TTA GAT ACC CTA-3′) and Mtrb1 (5′-TCT TAA TYC AAC ATC GAG GTC GCA A-3′) [58].

Molecular phylogenetic analysis
A multiple alignment of the nucleotide sequences was generated by the program MAFFT version 7.127b [59]. The nucleotide substitution model, GTR + I + G, was selected using the program ModelTest 2 [60,61]. The phylogenetic analyses were conducted by Bayesian (BA) and maximum-likelihood (ML) methods with the programs MrBayes v3.2.2 [62] and RAxML version 7.2.6 [63], respectively. In the BA analysis, in total 37,500 trees were obtained for each analysis (ngen = 50,000,000, samplefreq = 1,000, burn in = 12,501, temp = 0.2) and multiple independent runs were conducted to ensure the stable results. Posterior probabilities were calculated for each node by statistical evaluation in BA, whereas bootstrap values were obtained with 1000 replications in ML.

Results and discussion
Our diagnostic PCR survey of diverse stinkbugs and allied terrestrial heteropteran bugs, which represent 17 families, 77 genera, 108 species, 310 populations and 960 individuals, detected *Sodalis* symbionts from 17.6% (34/198) of the species, 11.0% (34/310) of the populations, and 17.2% (165/960) of the individuals (Table 1; Additional file 1). The infection frequencies were generally low and considerably variable among the different heteropteran groups: at the individual level, for example, 15.4% (4/26) in the Acanthosomatidae; 0.0% (0/33) in the Cydnidae; 5.6% (18/323) in the Pentatomidae; 0.0% (0/40) in the Plataspidae; 4.5% (11/247) in the Scutelleridae; 0.0% (0/30) in the Alydididae; 0.0% (0/45) in the Coreidae; and 0.0% (0/43) in the Blissidae (Table 1; Additional file 1). In the Urostylididae, by contrast, infection frequencies with
the *Sodalis* symbionts were exceptionally high: 100% (5/5) of the species, 100% (15/15) of the populations and 94.2% (130/138) of the individuals (Table 1; Additional file 1) [40]. In previous studies, 16S rRNA gene sequences of the *Sodalis* symbionts were determined for two scutellerid species *Cantao ocellatus* and *Eucoryses grandis* [38,39] and four urostylidid species *Urostylis annulicornis*, *Urostylis striicornis*, *Urostylis westwoodii* and *Urochela quadrinotata* [40]. In this study, we newly cloned and sequenced 16S rRNA gene of the *Sodalis* symbionts from the following heteropteran species: an acanthosomatid *Elasmucha putoni*; pentatomid *Aelia fieberi* (from two populations), *Dolycoris baccarum*, *Glaucias subpunctatus*, *Lelia decempunctata*, *Nezara antennata* (from three populations), *Palomena angulosa*, *Picromerus lewisi* and *Piezodorus hybneri*; a scutellerid *Poecilocoris lewisi*; and a rhopalid *Rhopalus sapporensis* (Figure 1). Molecular phylogenetic relationship of the *Sodalis* symbionts associated with the heteropteran bugs and other insects was inferred from the 16S rRNA gene sequences (Figure 2). The phylogenetic pattern indicated that (i) all the symbiont sequences were placed in the clade of *Sodalis*-allied bacteria with high statistical supports, (ii) the symbiont sequences within the same host species tended to be closely related to each other, (iii) nonetheless, the overall phylogenetic relationship of the symbiont sequences did not reflect the systematics of the host stinkbugs, and (iv) notably, the *Sodalis* symbionts of the urostylidid stinkbugs were not clustered with the *Sodalis* symbionts of the other stinkbug groups on the phylogeny.

The relatively low infection frequencies and the overall host-symbiont phylogenetic incongruence favor the hypothesis that the *Sodalis* symbionts are, in general, facultative associates for the heteropteran bugs, as *Wolbachia*, *Rickettsia*, *Spiroplasma*, etc. [41,64-66]. The majority of the plant-sucking heteropteran bugs harbor specific gut bacteria as the primary symbionts within the crypt cavities present in a posterior midgut region [1,67,68], which are important for normal growth, survival and reproduction of the host insects [69-80]. Probably, the majority of the *Sodalis* symbionts are, unlike the primary gut symbionts, not essential for their heteropteran hosts. On the other hand, it is conceivable, although speculative, that the *Sodalis* symbionts may play some substantial biological roles for their host stinkbugs in the Urostylididae. It deserves future studies what

Table 1 Detection of *Sodalis* symbionts from stinkbugs representing 17 families, 77 genera, 108 species, 310 populations and 960 individuals collected in Japan

| Superfamily family | Genus | Species | Population | Individual |
|-------------------|-------|---------|------------|------------|
| Pentatomoidea     |       |         |            |            |
| Acanthosomatidae  | 1/4 (25.0%) | 1/4 (25.0%) | 2/10 (20.0%) | 4/26 (15.4%) |
| Cydnidae          | 0/3 (0.0%) | 0/5 (0.0%) | 0/8 (0.0%) | 0/33 (0.0%) |
| Dinidoridae       | 0/1 (0.0%) | 0/1 (0.0%) | 0/5 (0.0%) | 0/12 (0.0%) |
| Parastrachiidae   | 0/1 (0.0%) | 0/1 (0.0%) | 0/1 (0.0%) | 0/10 (0.0%) |
| Pentatomidae      | 8/37 (21.6%) | 8/51 (15.7%) | 15/189 (7.9%) | 18/323 (5.6%) |
| Plataxidae        | 0/3 (0.0%) | 0/8 (0.0%) | 0/16 (0.0%) | 0/40 (0.0%) |
| Scutellaridae     | 3/7 (42.9%) | 3/8 (37.5%) | 3/29 (10.3%) | 11/247 (4.5%) |
| Urostylidae       | 2/2 (100%) | 5/5 (100%) | 15/15 (100%) | 130/138 (94.2%) |
| Coreoidea         |       |         |            |            |
| Alydidae          | 0/1 (0.0%) | 0/1 (0.0%) | 0/1 (0.0%) | 0/30 (0.0%) |
| Coreidae          | 0/9 (0.0%) | 0/13 (0.0%) | 0/19 (0.0%) | 0/45 (0.0%) |
| Ropalidae         | 1/1 (100%) | 1/1 (100%) | 1/1 (100%) | 1/1 (100%) |
| Lygaeoidea        |       |         |            |            |
| Berytidae         | 0/1 (0.0%) | 0/1 (0.0%) | 0/2 (0.0%) | 0/3 (0.0%) |
| Blissidae         | 0/1 (0.0%) | 0/1 (0.0%) | 0/5 (0.0%) | 0/43 (0.0%) |
| Lygaeidae         | 0/1 (0.0%) | 0/1 (0.0%) | 0/1 (0.0%) | 0/1 (0.0%) |
| Rhyparochromidae  | 0/3 (0.0%) | 0/3 (0.0%) | 0/3 (0.0%) | 0/3 (0.0%) |
| Pyrrhocoroidea    |       |         |            |            |
| Largidae          | 0/1 (0.0%) | 0/2 (0.0%) | 0/3 (0.0%) | 0/3 (0.0%) |
| Pyrrhocoridae     | 1/1 (100%) | 1/2 (50.0%) | 1/2 (50.0%) | 1/2 (50.0%) |
| Total             | 16/77 (20.8%) | 19/108 (17.6%) | 34/310 (11.0%) | 165/960 (17.2%) |
| Total without Urostylidae | 14/75 (18.7%) | 14/103 (13.6%) | 22/295 (7.5%) | 35/822 (4.3%) |
Figure 1 Stinkbugs associated with the *Sodalis* symbionts. (A) *Elasmucha putoni*. (B) *Aelia fieberi*. (C) *Dolycoris baccarum*. (D) *Glaçias subpunctatus*. (E) *Lelia decempunctata*. (F) *Nezara antennata*. (G) *Palomena angulosa*. (H) *Piezodorus hybneri*. (I) *Cantho ocellatus*. (K) *Eucorysses grandis*. (L) *Poecilocoris lewisi*. (M) *Urochela luteovaria*. (N) *Urochela quadrimaculata*. (O) *Urostylis annulicornis*. (P) *Urostylis striicornis*. (Q) *Urostylis westwoodii*. (R) *Rhoptalus sapporensis*. Photos by Toru Kawabe (A-D, G, I, L, M and R), Takahiro Hosokawa (E, F, J, K and Q), Joji Yokozeki (H), Gaku Miyake (N), Nahomi Kaiwa (O) and Yoshishige Shinogi (P).
biological roles, which are likely condition-dependent ones, the *Sodalis* symbionts play for their urostylidid hosts.

**Conclusions**

In conclusion, our results highlight that the *Sodalis* symbionts are facultative symbiotic bacteria commonly associated with diverse insects, as are *Wolbachia*, *Rickettsia*, *Spiroplasma*, *Cardinium*, *Arsenophonus*, and other widespread facultative symbionts. In this study, we exhaustively surveyed diverse stinkbugs in Japan, but, considering the recent report on the infection prevalence of the *Sodalis* symbiont in African populations of the coffee bug *Antestiopsis thunbergii* (Pentatomidae) [41], the occurrence of the *Sodalis* symbionts seems widespread among world’s stinkbugs and other insects. Future studies should focus on comprehensive survey of insect groups other than the heteropteran bugs, and also on effects and consequences of their infection to the host insects. Comparative studies on *Sodalis*-infected and uninfected host insects under the same genetic background combined with genomic and molecular biological analyses of the *Sodalis* symbionts will provide insights into ecological and evolutionary aspects of animal-microbe symbioses wherein the associations may range from free-living through facultative to obligate.

**Additional file**

**Additional file 1:** Stinkbug samples examined in this study and detection of *Sodalis* symbionts from the samples.

**Competing interests**

The authors declare that they have no competing interests.
Authors’ contributions
TH, NK and YK collected the insects and prepared the DNA samples. TH and NK performed PCR, cloning and sequencing of 16S rRNA gene of the Sodalis symbionts. TH and YM conducted molecular phylogenetic analysis. TF wrote the manuscript. All authors read and approved the final manuscript.

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