Bacterial Symbionts of a Devastating Coffee Plant Pest, the Stinkbug *Antestiopsis thunbergii* (Hemiptera: Pentatomidae)

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Stinkbugs of the genus *Antestiopsis*, so-called antestia bugs or variegated coffee bugs, are notorious pests of coffee plants in Africa. We investigated the symbiotic bacteria associated with *Antestiopsis thunbergii*, a major coffee plant pest in Rwanda. PCR, cloning, sequencing, and phylogenetic analysis of bacterial genes identified four distinct bacterial lineages associated with *A. thunbergii*: a gammaproteobacterial gut symbiont and symbionts representing the genera *Sodalis*, *Spiroplasma*, and *Rickettsia*. *In situ* hybridization showed that the gut symbiont densely occupied the lumen of midgut crypts, whereas the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont sparsely and sporadically infected various cells and tissues. Diagnostic PCR survey of 154 *A. thunbergii* individuals collected at 8 localities in Rwanda revealed high infection frequencies (100% for the gut symbiont, 51.3% for the *Sodalis* symbiont, 52.6% for the *Spiroplasma* symbiont, and 24.0% for the *Rickettsia* symbiont). These results suggest that the gut symbiont is the primary symbiotic associate of obligate nature for *A. thunbergii*, whereas the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont are the secondary symbiotic associates of facultative nature. We observed high coinfection frequencies, i.e., 7.8% of individuals with quadruple infection with all the symbionts, 32.5% with triple infections with the gut symbiont and two of the secondary symbionts, and 39.6% with double infections with the gut symbiont and any of the three secondary symbionts, which were statistically not different from the expected coinfection frequencies and probably reflected random associations. The knowledge of symbiotic microbiota in *A. thunbergii* will provide useful background information for controlling this devastating coffee plant pest.

Stinkbugs and allied bugs, belonging to the insect suborder Heteroptera within the order Hemiptera and consisting of over 40,000 described species worldwide, include a large number of devastating agricultural pests (1, 2). Symbiotic associations with bacteria are commonly found for these plant-sucking species, particularly those in the infraorder Pentatomomorpha. These insects possess a number of sacs or tubular outgrowths called crypts or ceca in a posterior region of the midgut, wherein a specific bacterial symbiont is harbored (3–5). When the gut symbionts are suppressed or eradicated experimentally, the symbiont-free insects tend to suffer retarded growth and elevated mortality (6–18), indicating important biological roles of the symbionts for their hosts. In addition to harboring these primary gut symbionts, many stinkbugs also harbor endosymbiotic bacteria of a facultative nature, such as *Wolbachia*, *Rickettsia*, *Sodalis*, and *Lariskella* species (19–22), although it has been poorly understood how these secondary symbionts affect their host's phenotype and fitness.

Stinkbugs of the genus *Antestiopsis* (Hemiptera: Heteroptera: Pentatomidae), often referred to as antestia bugs or variegated coffee bugs, are notorious as devastating pests of coffee plants in Africa and Southeast Asia (23). Adult and nymphal insects may feed on shoots and leaves of coffee plants but prefer to attack unripe coffee cherries. The attacks by antestia bugs not only physically damage the coffee cherries but also facilitate fungal infection of the fruits, causing coffee bean rot and significant yield loss (24, 25). Furthermore, particularly in East African countries, including Rwanda, Burundi, Tanzania, Zambia, and Kenya, coffee production is sometimes afflicted by an unfavorable flavor of coffee beans like potato peels (so-called “potato taste”), which is attributed to pyrazine compounds and downgrades the commercial value of the coffee products (26). For a long time, it has been hypothesized

that the development of potato taste is correlated with infestation by antestia bugs. Researchers have suspected that insect-derived chemicals, insect-vectored pathogens, or insect-borne microbes may induce the potato taste in the infested coffee cherries, but no convincing proof has been provided (27). Thus far, symbiotic bacteria associated with antestia bugs have been poorly characterized.

In this study, we investigated the symbiotic microbiota of the antestia bug *Antestiopsis thunbergii* in Rwanda, one of the focal areas suffering the potato taste problem. We identified a midgut-associated obligate gammaproteobacterial symbiont and three facultative symbionts representing the bacterial genera *Sodalis*, *Spiroplasma*, and *Rickettsia* and surveyed their infection prevalences in more than 150 individuals originating from 8 geographic populations.

MATERIALS AND METHODS

Insect materials. Coffee bugs were collected from *Coffea arabica* coffee trees at different localities in Rwanda from 2012 to 2014 (Table 1 and Fig. 1). The insects were morphologically identified as *Antestiopsis thunbergii* ghesquierei Carayon. The samples were preserved in 95% ethanol.

DNA extraction, PCR, cloning, and sequencing. Some of the preserved insects were subjected to dissection of midgut and ovary prepara-

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TABLE 1 Samples of *Antestiopsis thunbergii* and their infection frequencies with symbiotic bacteria

Collection locality (no. on map in Fig. 1) ^a	Collection date(s)	No. of infected insects/no. examined (infection rate [%])			
		Gut symbiont	<i>Sodalis</i> symbiont	<i>Spiroplasma</i> symbiont	<i>Rickettsia</i> symbiont
Gakenke, Gakenke, NR (1)	16 Jan 2012, 11 Apr 2012, 23 Dec 2013	16/16 (100)	16/16 (100)	13/16 (81.3)	7/16 (43.8)
Ruli, Rulindo, NR (2)	17 Dec 2013	15/15 (100)	13/15 (86.7)	10/15 (66.7)	1/15 (6.7)
Nyamagabe, Nyamagabe, SR (3)	12 Apr 2012	11/11 (100)	3/11 (27.3)	9/11 (81.8)	0/11 (0.0)
Karubanda, Huye, SR (4)	06 Jan 2014	29/29 (100)	10/29 (34.5)	8/29 (27.6)	17/29 (58.6)
Maraba, Huye, SR (5)	02 Jan 2014	11/11 (100)	5/11 (45.5)	9/11 (81.8)	4/11 (36.4)
Mubuga, Karongi, WR (6)	14 Dec 2013	32/32 (100)	21/32 (65.6)	16/32 (50.0)	4/32 (12.5)
Karengera/Mwezi, Nyamasheke, WR (7)	19 Dec 2013	32/32 (100)	7/32 (21.9)	12/32 (37.5)	2/32 (6.3)
Mahembe, Karongi, WR (8)	12 Dec 2013	8/8 (100)	4/8 (50.0)	4/8 (50.0)	2/8 (25.0)
Total		154/154 (100)	79/154 (51.3)	81/154 (52.6)	37/154 (24.0)

^a Names of sectors, districts, and provinces are shown. NR, SR, and WR indicate Northern Province, Southern Province, and Western Province of Rwanda, respectively.

tions, which were individually subjected to DNA extraction, PCR amplification of a 1.5-kb region of the bacterial 16S rRNA gene with primers 16SA1 and 16SB1 (28), cloning of the PCR product, and sequencing of the clones as described previously (29). For phylogenetic analysis, a 1.6-kb region of the protein-encoding bacterial *groEL* gene was amplified by PCR with primers Gro-F1 and Gro-R1, cloned, and sequenced as described previously (30). These primer sequences are listed in Table 2.

Molecular phylogenetic and evolutionary analyses. Multiple alignments were generated by the program MAFFT version 7.127b (31). Ambiguously aligned nucleotide sites were manually removed. Phylogenetic analyses were conducted by three methods: maximum likelihood, maximum parsimony, and neighbor-joining methods. The nucleotide and amino acid substitution models were selected using MEGA version 5.2.2 (32). Maximum likelihood trees were generated by the PhyML 3.0 program (33), while maximum parsimony and neighbor-joining trees were constructed by using MEGA version 5.2.2. Bootstrap values were obtained with 1,000 replications. Relative-rate tests were performed using the program RRTree (34).

Fluorescence *in situ* hybridization. Fluorescence *in situ* hybridization was performed essentially as previously described, with some modifications (35, 36). The insect samples preserved in ethanol were washed with 80% and 50% ethanol for 10 min each. Then, the abdominal tissues

were dissected and fixed in Carnoy's solution (ethanol-chloroform-acetic acid, 6:3:1) for several hours. The fixed samples were thoroughly washed with 100% ethanol, cleared through an ethanol-xylene series, and embedded in paraffin. After the paraffin blocks were processed into serial 3- to 4-μm sections and mounted onto glass slides (MAS-coated Superfrost; Matsunami Glass Ind., Ltd.), the sections were dewaxed through a xylene-ethanol series and air dried. After being rinsed twice with phosphate-buffered saline (PBS), the samples were treated with 0.1 mg/ml pepsin in 0.01 N HCl for 15 min at 37°C, washed with 100% ethanol twice, and air dried. About 150 μl of hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide) was applied onto the samples, with 100 nM concentrations (each) of the following fluorochrome-labeled oligonucleotide probes for targeting the symbionts: AtGS419 (for the gut symbiont), Sod1233 (for the *Sodalis* symbiont), Spr403 (for the *Spiroplasma* symbiont), and Ric1071 (for the *Rickettsia* symbiont). These probe sequences are listed in Table 2. The slides were covered with coverslips and incubated in a humidified chamber at 35°C overnight. For the removal of nonspecific probe binding, the samples were washed in the hybridization buffer without the probes for 1 h at 42°C. After being thoroughly washed, the samples were mounted in Slow-fade antifade solution (Invitrogen) supplemented with 0.25 μM SYTOX green or SYTOX orange (Invitrogen) and observed under a laser confocal microscope (Pascal 5; Carl Zeiss).

Diagnostic PCR. The following primers were used for specific PCR detection of 16S rRNA gene of the symbionts: antestia175F and antestia419R (targeting a 0.26-kb region of the gut symbiont), sodalis370F and 16SB1 (targeting a 1.1-kb region of the *Sodalis* symbiont) (20), 16SA1 and TKSSsp (targeting a 0.49-kb region of the *Spiroplasma* symbiont) (37), and 16SA1 and Rick16SR (targeting a 0.20-kb region of the *Rickettsia* symbiont) (38). The sequences of these primers are listed in Table 2. The PCR temperature profile was 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 53°C for the gut symbiont gene or 55°C for the genes of the other symbionts for 1 min, and 72°C for 1.5 min for all the symbiont genes.

Statistics. Expected coinfection frequencies were calculated based on the infection frequency of each symbiont. Differences between expected coinfection frequencies and observed coinfection frequencies were tested with Fisher's exact test using the software R version 2.12.1 (39).

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA gene and the *groEL* gene have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB809070 to AB809076.

RESULTS AND DISCUSSION

Bacterial 16S rRNA gene sequences from dissected tissues of *A. thunbergii*. Midgut and ovary preparations were dissected from the fixed specimens of two adult females collected at Gakenke,

FIG 1 Localities in Rwanda where the samples of *Antestiopsis thunbergii* were collected. 1, Gakenke; 2, Ruli; 3, Nyamagabe; 4, Karubanda; 5, Maraba; 6, Mubuga; 7, Karengera/Mwezi; 8, Mahembe. (Template map is from Wikimedia Commons [http://commons.wikimedia.org/wiki/File:Rwanda_map_blank.png].)

TABLE 2 Primers and probes used in this study

Primer type/probe and target bacteria	Target gene	Primer/probe name	Primer/probe sequence	Reference
Primers for PCR, cloning, and sequencing of bacterial genes (bacteria generally)	16S rRNA gene	16SA1	5'-AGAGTTTGATCMTGGCTCAG-3'	28
		16SB1	5'-TACGGYTACCTTGTTACGACTT-3'	28
	groEL	Gro-F1	5'-ATGGCAGCWAAAGACGTAAATTYGG-3'	30
		Gro-R1	5'-TTACATCATKCCGCCCATGC-3'	30
Primers for diagnostic PCR detection of bacterial symbionts				
Gut symbiont	16S rRNA gene	antestia175F	5'-GGACCTTTAGGCCTCAT-3'	This study
		antestia419R	5'-ATCTTACCGCCTTCCTC-3'	This study
Sodalis symbiont	16S rRNA gene	sodalis370F	5'-CGRTRGCGTTAAAYAGCGC-3'	20
		16SB1	5'-TACGGYTACCTTGTTACGACTT-3'	28
Spiroplasma symbiont	16S rRNA gene	16SA1	5'-AGAGTTTGATCMTGGCTCAG-3'	28
		TKSSsp	5'-TAGCCGTGGCTTTCTGGTAA-3'	37
Rickettsia symbiont	16S rRNA gene	16SA1	5'-AGAGTTTGATCMTGGCTCAG-3'	28
		Rick16SR	5'-CATCCATCAGCGATAAATCTTTC-3'	38
Probes for in situ hybridization targeting 16S rRNA of bacterial symbionts				
Gut symbiont		AtGS419	5'-Alexa 647-ATCTTACCGCCTTCCTC-3'	This study
Sodalis symbiont		Sod1233	5'-Alexa 488-GCTGACTCTCGCAGATC-3'	This study
Spiroplasma symbiont		Spr403	5'-Alexa 555-TACTTACTGTTCTTCCCTTACA-3'	This study
Rickettsia symbiont		Ric1071	5'-Alexa 555-CTTATAGTTCCCGGCATTAC-3'	This study

Northern Province, Rwanda (female N1 and female N2), which were subjected to DNA extraction, PCR, cloning, and sequencing of the bacterial 16S rRNA gene. Here it should be noted that, since the midgut and the ovary were intermingled and stuck to each other in the fixed specimens, cross contamination between the dissected tissues was expected to some extent. Five clones from the midgut of female N1 and six clones from the midgut of female N2 were sequenced, and these exhibited the same 1,465-bp nucleotide sequence (type A), with the highest BLAST hit to the 16S rRNA gene sequence of *Kluyvera cryocrescens* (*Gammaproteobacteria: Enterobacteriaceae*) (96.2% [1,410/1,465 bp] identity; GenBank accession number [AY946284](#)). Eight clones from the ovary of female N1 and 14 clones from the ovary of female N2 were also sequenced, and these yielded four distinct 16S rRNA gene sequences: a 1,464-bp sequence (type B; three clones from female N1 and two clones from female N2) with the highest BLAST hit to the 16S rRNA gene sequence of a *Sodalis*-allied symbiont (*Gammaproteobacteria: Enterobacteriaceae*) of the weevil *Curculio sikimensis* (99.6% [1,448/1,454 bp] identity; GenBank accession number [AB517595](#)), a 1,442-bp sequence (type C; two clones from female N1 and two clones from female N2) with the highest BLAST hit to the 16S rRNA gene sequence of *Spiroplasma leucomae* (*Mollicutes: Spiroplasmataceae*) from the moth *Leucoma salicis* (99.3% [1,432/1,442 bp] identity; GenBank accession number [AB681166](#)), a 1,421-bp sequence (type D; three clones from female N1 only) with the highest BLAST hit to *Rickettsia felis* (*Alphaproteobacteria: Rickettsiaceae*) (99.1% [1,408/1,421 bp] identity; GenBank accession number [CP000053](#)), and 10 clones of the type A sequence (from female N2 only). Furthermore, midgut and ovary preparations were also dissected from an adult female collected at Gakenke (female N3), and a midgut preparation was dissected from an adult female collected at Nyamagabe, Southern Province, Rwanda (female S1, whose ovary was not developed). These samples were similarly subjected to DNA extraction, PCR,

cloning, and sequencing of the bacterial 16S rRNA gene, which yielded the following sequences: six type A sequences and a type C sequence from the midgut of female N3, three type C sequences and five type D sequences from the ovary of female N3, and eight type A sequences from the midgut of female S1. The type A, B, C, and D sequences were identical across the insect individuals and across the collection localities that we examined.

Molecular phylogenetic analysis of bacterial 16S rRNA gene sequences from *A. thunbergii*. The 16S rRNA gene sequences were subjected to molecular phylogenetic analyses together with allied bacterial sequences retrieved from the DNA databases. The type A sequence comprised a distinct lineage in the *Gammaproteobacteria*, which clustered with gut symbionts of the scutellerid stinkbug *Eucorysses grandis* and the cydnid stinkbug *Macroscytus japonensis*, and also with *Kluyvera cryocrescens* and *Enterobacter ludwigii*, although statistical support for the grouping was low (Fig. 2; see Fig. S1 in the supplemental material). The type B sequence was placed in the clade of *Sodalis glossinidius* and allied insect symbionts in the *Gammaproteobacteria*, which had high statistical support (Fig. 2; see Fig. S1). The type C sequence was closely related to the sequences of *Spiroplasma* spp. associated with various insects and plants in the *Mollicutes*, and this grouping was supported by 100% statistical values (Fig. 2; see Fig. S2). The type D sequence was allied with *Rickettsia felis* and other *Rickettsia* species associated with ticks and insects, belonging to the clade of the genus *Rickettsia* with high statistical support (Fig. 2; see Fig. S3). Here we refer to the symbionts representing the type A, type B, type C, and type D sequences as the gut symbiont, *Sodalis* symbiont, *Spiroplasma* symbiont, and *Rickettsia* symbiont of *A. thunbergii*, respectively.

Bacterial *groEL* gene sequences from dissected tissues of *A. thunbergii*. Additionally, a 1.6-kb region of the bacterial *groEL* gene was amplified, cloned, and sequenced from the midgut and ovary preparations of female N1 and female N2. All six clones

FIG 2 Phylogenetic placement of the gut symbiont, the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont associated with *Antestiopsis thunbergii* on the basis of 16S rRNA gene sequences. A maximum likelihood phylogeny inferred from 1,223 aligned nucleotide sites is shown, while maximum parsimony and neighbor-joining analyses gave substantially the same results. Bootstrap values of 50% or higher are shown at the nodes in the order of maximum likelihood/maximum parsimony/neighbor joining. Asterisks indicate support values lower than 50%. Symbionts of stinkbugs are highlighted by boldface type, and symbionts of *A. thunbergii* are further underlined. Sequence accession numbers are shown in brackets. The scale bar indicates the branch length measured by the number of substitutions per site.

from the midgut of female N1, all seven clones from the midgut of female N2, and four of eight clones from the ovary of female N2 exhibited the same 1,607-bp nucleotide sequence. The remaining four clones from the ovary of female N2 showed the same 1,583-bp nucleotide sequence.

Molecular phylogenetic analysis of bacterial *groEL* gene sequences from *A. thunbergii*. Molecular phylogenetic analysis of

translated amino acid sequences revealed that the former sequence predominant in the midgut (the 1,607-bp sequence) clustered with a gut symbiont of the scutellerid stinkbug *Cantao ocellatus*, whereas the latter minor sequence detected from the ovary (the 1,583-bp sequence) was placed in a highly supported clade with *Sodalis glossinidius* and allied insect symbionts (see Fig. S4 in the supplemental material). These results indicate that the major and minor *groEL* gene sequences are derived from the gut symbiont and the *Sodalis* symbiont of *A. thunbergii*, respectively.

***In vivo* localization of the *A. thunbergii* symbionts.** Midgut and ovary preparations dissected from adult females of *A. thunbergii* of the population of the Northern Province of Rwanda were subjected to fluorescence *in situ* hybridization using specific probes targeting 16S rRNA of each of the symbionts. Strong signals of the gut symbiont were detected exclusively in the lumen of the midgut crypts at very high densities (Fig. 3A and B), while signals of the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont were sparse, sporadic, and observed in various cells and tissues at low densities (Fig. 3C to F). The signals of the gut symbiont were observed neither in the ovary nor in tissues other than the midgut crypts (data not shown). Hence, the sporadic detection of gut symbiont sequences from the dissected ovaries was quite likely due to contamination of midgut tissues during dissection of the ethanol-preserved insects.

Infection prevalence of the symbionts in field *A. thunbergii* populations. In total, we collected 154 *A. thunbergii* individuals from coffee orchards at eight localities across the Northern, Southern, and Western Provinces of Rwanda and surveyed the infection prevalences of these symbionts by diagnostic PCR detection. The gut symbiont was detected from all the insects examined (Table 1), indicating that the gut symbiont residing in the midgut crypts is the primary symbiont of *A. thunbergii*. In contrast, the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont exhibited partial infection frequencies of 51.3%, 52.6%, and 24.0%, respectively (Table 1). These infection patterns and the sporadic *in vivo* localizations (Fig. 3C to F) suggest that these symbionts are secondary symbiotic associates of facultative nature for *A. thunbergii*.

Coinfection patterns of the *A. thunbergii* symbionts. Table 3 summarizes coinfection patterns of the gut symbiont, the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont in the field populations of *A. thunbergii*. While all 154 insects were infected with the gut symbiont, 31 insects (20.1%) were singly infected with the gut symbiont only, 61 insects (39.6%) were doubly infected with the gut symbiont and any of the three secondary symbionts, 50 insects (32.5%) were triply infected with the gut symbiont and two of the secondary symbionts, and 12 insects (7.8%) were quadruply infected with all the symbionts. The observed coinfection frequencies were statistically not deviated from the expected coinfection frequencies under the assumption of random associations (Table 3). These patterns suggest that interactions between the coinfecting symbionts are apparently neither agonistic nor synergistic to each other, although this idea should be experimentally verified by comparing the host insects infected with different combinations of the symbionts under controlled genetic backgrounds.

Conclusion and perspective. In this study, we identified the gut symbiont as the primary symbiotic associate of *A. thunbergii*, as in allied stinkbugs of the family Pentatomidae (3–5). Molecular evolutionary analyses revealed that the 16S rRNA and *groEL* genes

FIG 3 Localization of the bacterial symbionts in the abdomen of *Antestiopsis thunbergii*. In the fluorescence *in situ* hybridization (FISH) images, blue, green, and red signals indicate insect nuclear DNA, the gut symbiont, and the *Sodalis*, *Spiroplasma*, or *Rickettsia* symbiont, respectively. (A) FISH of a tissue section of the posterior midgut, in which crypt cavities are filled with the gut symbiont cells. (B) FISH of a tissue section of the posterior midgut from another insect, wherein the *Sodalis* symbiont cells are sporadically located in gut epithelia and fat body cells in addition to the gut symbiont cells in crypt cavities. (C) The *Sodalis* symbiont cells in a fat body cell. (D) FISH of a tissue section of the posterior midgut region without staining of the gut symbiont, wherein the *Spiroplasma* symbiont cells are sporadically detected in fat body cells. (E) The *Spiroplasma* symbiont cells in a fat body cell. (F) The *Rickettsia* symbiont cells in midgut epithelial cells. Abbreviations: fb, fat body; G, gut symbiont; mgc, midgut crypt; R, *Rickettsia* symbiont; Sd, *Sodalis* symbiont; Sp, *Spiroplasma* symbiont.

of the gut symbiont are evolving significantly faster than those of related free-living bacteria (Table 4). Together with 100% infection frequencies in the field host populations (Table 1), these patterns strongly suggest an obligate host-symbiont association maintained over evolutionary time. Biological roles of the gut symbiont for *A. thunbergii* are to be established by comparing fitness parameters and other phenotypes between experimentally

generated symbiotic and aposymbiotic insects under controlled genetic backgrounds. On account of previous studies on other pentatomid stinkbugs and their gut symbionts (6, 11–13, 18), it seems quite likely that the gut symbiont is beneficial for growth, survival, and reproduction of *A. thunbergii*. If the gut symbiont is essential for *A. thunbergii*, it can be a potential target for controlling this devastating pest of coffee plants.

TABLE 3 Coinfection patterns of *Antestiopsis thunbergii* with the gut symbiont, the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont

Locality	No. of insects infected/no. examined (infection rate [%]) ^a							
	G	G, Sd	G, Sp	G, R	G, Sd, Sp	G, Sd, R	G, Sp, R	G, Sd, Sp, R
Gakenke	0/16 (0)	2/16 (12.5)	0/16 (0)	0/16 (0)	7/16 (43.8)	1/16 (6.3)	0/16 (0)	6/16 (37.5)
Ruli	0/15 (0)	5/15 (33.3)	1/15 (6.6)	0/15 (0)	8/15 (53.3)	0/15 (0)	1/15 (6.6)	0/15 (0)
Nyamagabe	2/11 (18.2)	0/11 (0)	6/11 (54.5)	0/11 (0)	3/11 (27.3)	0/11 (0)	0/11 (0)	0/11 (0)
Karubanda	4/29 (13.8)	5/29 (17.2)	2/29 (6.9)	8/29 (27.6)	1/29 (3.4)	4/29 (13.8)	5/29 (17.2)	0/29 (0)
Maraba	1/11 (9.1)	1/11 (9.1)	4/11 (36.4)	0/11 (0)	1/11 (9.1)	0/11 (0)	1/11 (9.1)	3/11 (27.3)
Mubuga	6/32 (18.8)	8/32 (25.0)	4/32 (12.5)	0/32 (0)	10/32 (31.3)	2/32 (6.3)	1/32 (3.1)	1/32 (3.1)
Karengera/Mwezi	16/32 (50)	4/32 (12.5)	8/32 (25)	0/32 (0)	2/32 (6.3)	0/32 (0)	1/32 (3.1)	1/32 (3.1)
Mahembe	2/8 (25)	1/8 (12.5)	1/8 (12.5)	1/8 (12.5)	2/8 (25)	0/8 (0)	0/8 (0)	1/8 (12.5)
Total	31/154 (20.1)	26/154 (16.9)	26/154 (16.9)	9/154 (5.8)	34/154 (22.1)	7/154 (4.5)	9/154 (5.8)	12/154 (7.8)
Expected % value ^b	17.5	18.5	19.5	5.5	20.5	5.8	6.1	6.5

^a G, infected with the gut symbiont; Sd, infected with the *Sodalis* symbiont; Sp, infected with the *Spiroplasma* symbiont; R, infected with the *Rickettsia* symbiont.

^b Calculated from the infection frequencies shown in Table 1. The calculated values were not significantly different from the expected values (Fisher's exact test, $P > 0.05$).

TABLE 4 Relative rate tests for comparing the molecular evolutionary rates of 16S rRNA and *groEL* gene sequences between the lineages of the gut symbiont of *Antestiopsis thunbergii* and allied free-living gammaproteobacteria^c

Gene	Lineage 1	Lineage 2	Outgroup	K1 ^a	K2 ^b	K1 – K2	K1/K2
16S rRNA	Gut symbiont of <i>Antestiopsis thunbergii</i> (AB809070)	<i>Enterobacter ludwigii</i> (EF175735)	<i>Yersinia pestis</i> (AE009952)	0.074	0.056	0.018	1.32
<i>groEL</i>	Gut symbiont of <i>Antestiopsis thunbergii</i> (AB809075)	<i>Erwinia pyrifoliae</i> (NC_017390)	<i>Yersinia pestis</i> (AE009952)	0.129	0.089	0.040	1.45

^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 The molecular evolutionary rates were significantly faster in the lineage of the gut symbiont than in the lineage of allied free-living gammaproteobacteria for both the 16S rRNA gene ($P < 0.01$) and the *groEL* gene ($P < 0.01$). These P values were generated using the program RRTree (34). The analysis was performed using 1,233 nucleotide sites of the 16S rRNA gene sequence and 505 amino acid sites inferred from the *groEL* gene sequence, respectively.

We also identified the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont as the secondary symbiotic associates of a facultative nature for *A. thunbergii*. It should be noted that, considering the relatively small sample sizes subjected to cloning and sequencing of the bacterial 16S rRNA gene, other facultative symbionts may remain undetected in natural populations of *A. thunbergii*. The high levels of symbiont coinfections in *A. thunbergii* (Table 3) are striking but not exceptional: a number of recent studies have revealed frequent coinfections with multiple symbiotic bacteria in natural insect populations (40–43).

It has been reported that in various insects, some facultative symbionts significantly contribute to the fitness of their hosts by conferring resistance to parasites and pathogens, tolerance against environmental stresses, suppressed capacity for vectoring animal/plant pathogens, and so on (44, 45), while other facultative symbionts affect reproduction of their hosts in such ways that drive their own infection into hosts' populations (46, 47). Such phenotypic aspects of the *Sodalis* symbiont, the *Spiroplasma* symbiont, and the *Rickettsia* symbiont in *A. thunbergii* may be of interest to applied scientists and deserve future studies (48–50). The causal agent of potato taste associated with *Antestiopsis*-infested coffee cherries has not been identified, and the possibility that these symbiotic bacteria may be involved in the development of potato taste in some way is worth pursuing.

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