The ISME Journal (2013) 7, 1378–1390

www.nature.com/ismej

ORIGINAL ARTICLE

Diversification of endosymbiosis: replacements, co-speciation and promiscuity of bacteriocyte symbionts in weevils

Hirokazu Toju[1,2,3], Akifumi S Tanabe[2,4], Yutaka Notsu[5], Teiji Sota[6] and Takema Fukatsu[1]
[1]*Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan;* [2]*Graduate School of Global Environmental Studies, Kyoto University, Sakyo, Kyoto, Japan;* [3]*Graduate School of Human and Environmental Studies, Kyoto University, Sakyo, Kyoto, Japan;* [4]*Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan;* [5]*Kanagawa Prefectural Zama Special Education School, Zama, Japan and* [6]*Graduate School of Science, Kyoto University, Sakyo, Kyoto, Japan*

The processes and mechanisms underlying the diversification of host–microbe endosymbiotic associations are of evolutionary interest. Here we investigated the bacteriocyte-associated primary symbionts of weevils wherein the ancient symbiont *Nardonella* has experienced two independent replacement events: once by *Curculioniphilus* symbiont in the lineage of *Curculio* and allied weevils of the tribe Curculionini, and once by *Sodalis*-allied symbiont in the lineage of grain weevils of the genus *Sitophilus*. The *Curculioniphilus* symbiont was detected from 27 of 36 Curculionini species examined, the symbiont phylogeny was congruent with the host weevil phylogeny, and the symbiont gene sequences exhibited AT-biased nucleotide compositions and accelerated molecular evolution. These results suggest that the *Curculioniphilus* symbiont was acquired by an ancestor of the tribe Curculionini, replaced the original symbiont *Nardonella*, and has co-speciated with the host weevils over evolutionary time, but has been occasionally lost in several host lineages. By contrast, the *Sodalis*-allied symbiont of *Sitophilus* weevils exhibited no host–symbiont co-speciation, no AT-biased nucleotide compositions and only moderately accelerated molecular evolution. These results suggest that the *Sodalis*-allied symbiont was certainly acquired by an ancestor of the *Sitophilus* weevils and replaced the original *Nardonella* symbiont, but the symbiotic association must have experienced occasional re-associations such as new acquisitions, horizontal transfers, replacements and/or losses. We detected *Sodalis*-allied facultative symbionts in populations of the Curculionini weevils, which might represent potential evolutionary sources of the *Sodalis*-allied primary symbionts. Comparison of these newcomer bacteriocyte-associated symbiont lineages highlights potential evolutionary trajectories and consequences of novel symbionts after independent replacements of the same ancient symbiont.
The ISME Journal (2013) **7,** 1378–1390; doi:10.1038/ismej.2013.27; published online 28 February 2013
Subject Category: microbe-microbe and microbe-host interactions
Keywords: Curculionidae; *Curculioniphilus*; *Sodalis*-allied symbiont; *Nardonella*; host-symbiont co-evolution; symbiont replacement

Introduction

Insects embrace some 1 000 000 described species on the earth (Stork, 2003), and many of them host phylogenetically diverse microbial symbionts (Buchner, 1965; Bourtzis and Miller, 2003). Some symbionts like *Wolbachia* in diverse insects are of facultative nature, not essential for their hosts, and usually localized in a broad array of cells and tissues (Werren *et al.*, 2008; Oliver *et al.*, 2010). Other symbionts like *Buchnera* in aphids and *Wigglesworthia* in tsetse flies are of obligate nature, essential for survival and reproduction of their hosts, and often localized in specialized cells called bacteriocytes (Baumann, 2005; Moran *et al.*, 2008).

In general, these symbionts are stably maintained through host generations by vertical transmission, but the evolutionary trajectories may be markedly different between the facultative ones and the obligate ones. In the facultative symbiotic association, the symbiont phylogeny is generally incongruent with the host phylogeny, indicating horizontal transfers and/or new acquisitions of the symbionts at considerable frequencies in their

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
Received 4 November 2012; revised 9 January 2013; accepted 23 January 2013; published online 28 February 2013

evolutionary course. In the obligate symbiotic associations, by contrast, the symbiont phylogeny often mirrors the host phylogeny, reflecting strict vertical symbiont transmission and host–symbiont co-speciation over evolutionary time. Furthermore, these obligate symbionts tend to exhibit remarkable evolutionary patterns such as AT-biased nucleotide composition, accelerated molecular evolution and reduced genome size, which are attributable to the stable and nutrition-rich habitat for the symbionts and also attenuated purifying selection because of small population size and strong bottleneck associated with the lifestyle of the vertically transmitted symbionts (Wernegreen, 2002; Moran *et al*., 2008).

Such obligate host–symbiont associations may be evolutionarily stable, but acquisitions and/or replacements of obligate symbionts must have taken place occasionally. For example, aphids, mealybugs, whiteflies and psyllids constitute a monophyletic group in the order Hemiptera, and are commonly associated with specific bacterial symbionts within the bacteriocytes: *Buchnera* in aphids, *Tremblaya* in mealybugs, *Portiera* in whiteflies and *Carsonella* in psyllids. However, these symbionts are phylogenetically distinct between the insect groups, suggesting acquisitions and/or replacements of the bacteriocyte-associated symbionts during their diversification (Baumann, 2005; Moran *et al.*, 2008). Within aphids, although the majority of over 4000 species are associated with *Buchnera*, about 20 species representing three genera of the tribe Cerataphidini have lost *Buchnera* and acquired yeast-like fungal symbionts in their body cavity (Fukatsu and Ishikawa, 1992, 1996; Fukatsu *et al.*, 1994; Hongoh and Ishikawa, 2000).

Weevils constitute the most species-rich metazoan family Curculionidae with over 51 000 described species worldwide (Farrell, 1998; McKenna *et al.*, 2009), which provide an ideal opportunity to investigate the evolutionary processes underlying acquisitions, replacements and diversification of novel host–symbiont associations. The bacteriocyte-associated *Nardonella* symbionts are found from diverse weevil subfamilies including the Dryophthorinae and the Molytinae, have strictly co-speciated with their hosts for over 125 million years, and exhibit highly AT-biased nucleotide compositions and accelerated molecular evolution that are typical of ancient insect symbionts of obligate nature (Lefèvre *et al*., 2004; Conord *et al.*, 2008; Hosokawa and Fukatsu, 2010). On the other hand, the bacteriocyte-associated *Sodalis*-allied symbionts are restricted to a few grain weevil species of the genus *Sitophilus* (Heddi and Nardon, 2005), and the bacteriocyte-associated *Curculioniphilus* symbionts were recently described from several seed-infesting weevils of the genus *Curculio* (Toju *et al.*, 2010). *Nardonella* symbionts, *Sodalis*-allied symbionts and *Curculioniphilus* symbionts form distinct clades within the *gammaproteobacteria*, respectively (Toju *et al*., 2010). The weevil genera *Sitophilus* and *Curculio* constitute distinct lineages within the family Curculionidae: *Sitophilus* is placed within the Dryophthorinae whereas *Curculio* clusters with the Molytinae (McKenna *et al*., 2009). These phylogenetic patterns indicate that, in the evolutionary course of the weevils, the ancient bacteriocyte-associated symbiont *Nardonella* has experienced at least two independent replacement events: once by *Sodalis*-allied symbiont in the lineage of *Sitophilus* weevils, and once by *Curculioniphilus* symbiont in the lineage of *Curculio* and allied weevils. It is of interest what evolutionary patterns are observed in the phylogenetically distinct novel symbionts and their hosts, and how replacements to the novel symbionts have affected ecological traits of their hosts such as food plant utilization.

In this study, we collected 36 weevil species representing the genus *Curculio* and allied genera of the tribe Curculionini, and characterized their symbiotic bacteria on the basis of 16S ribosomal RNA (rRNA) gene sequences. *Curculioniphilus* symbionts were detected from 27 of the 36 weevil species, and we analyzed their molecular evolutionary patterns, host–symbiont co-speciation and correlation with food plants of their hosts. Then, we retrieved gene sequences of three *Sitophilus* weevil species and their *Sodalis*-allied symbionts from the DNA databases, and performed similar molecular phylogenetic analyses. Comparison of these newcomer bacteriocyte-associated symbiont lineages highlighted contrasting evolutionary trajectories and consequences of the novel symbionts that independently replaced the same ancient symbiont.

Materials and methods

Curculionini weevils

The Curculionini is a tribe of weevils within the subfamily Curculioninae (Coleoptera: Curculionidae). Among 18 genera of the tribe, the genus *Curculio* is the largest and embraces about 350 described species worldwide (Alonso-Zarazaga and Lyal, 1999; Hughes and Vogler, 2004a). In most of the species whose ecological information is available, their host plant range is limited to a small number of species within a single plant genus (Morimoto, 1960, 1962; Hayashi *et al*., 1984; Fujimoto, 2004; Hughes and Vogler, 2004b). In tropical and subtropical regions, diverse Curculionini weevils are associated with figs (Moraceae), while in Europe and North America, they are infesting hard seeds such as acorns and chestnuts (Fagaceae), hazelnuts and birches (Betulaceae) and pecans and walnuts (Juglandaceae) (Hughes and Vogler, 2004a, 2004b). In Japan, Curculionini weevils utilize host plants from such families as the Caprifoliaceae, Elaeagnaceae, Fabaceae, Lauraceae, Styracaceae, Theaceae and Ulmaceae, in addition to the Fagaceae, Betulaceae and Moraceae (Morimoto, 1960, 1962; Hayashi

et al., 1984; Fujimoto, 2004; H Toju, unpublished; Y Notsu, unpublished). Although most species are seed feeders, some small-sized species feed on insect galls formed on Fagaceae or Salicaceae plants (Hayashi *et al*., 1984; Sugiura, *et al*., 2002; Fujimoto, 2004). These gall feeders are currently classified to three genera *Archarius*, *Curculio* and *Koreoculio* (Fujimoto, 2004), and a molecular phylogenetic study suggested that the gall-feeding habit evolved at least twice in the Curculionini (Hughes and Vogler, 2004a).

Sampling and DNA extraction
In total, 36 weevil species representing 5 known and 1 unidentified genera of the tribe Curculionini were collected from Japan and Southeast Asia (Supplementary Table S1). The specimens were preserved in 99% ethanol or acetone (Fukatsu 1999). The specimens were individually subjected to DNA extraction using QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands). The quality of the DNA samples was confirmed by PCR amplification of a 0.8-kb fragment of mitochondrial cytochrome oxidase subunit I gene of the insect using primers C1-J-2183 and L2-N-3014 (Supplementary Table S2).

DNA cloning, genotyping and sequencing
A 1.5-kb fragment of bacterial 16S rRNA gene was amplified by PCR with primers 16SA1 and 16SB1 (Supplementary Table S2), and the PCR products were subjected to cloning, restriction fragment length polymorphism genotyping, and sequencing as previously described (Fukatsu and Nikoh, 1998). Accession numbers for the 16S rRNA gene clones are listed in Supplementary Table S3. Two mitochondrial genes (0.8-kb fragments of cytochrome oxidase subunit I; 0.4-kb fragments of cytochrome *b*) and three nuclear genes (0.4-kb fragments of elongation factor 1α, 0.7-kb fragments of 28S rRNA and 0.4-kb fragments of phosphoglycerate mutase) were amplified by PCR with primers listed in Supplementary Table S2, and cloned and sequenced as described above. Accession numbers for the sequences are listed in Supplementary Table S4.

Diagnostic PCR detection of Curculioniphilus *symbiont*
Specific primers Clp_Unv_456F (5′-GGGTTGTAAA GCACTTTCAGT-3′) and Clp_Unv_560R (5′-AYARRC CGCCTACGYACT-3′) targeting a 0.1-kb fragment of *Curculioniphilus* 16S rRNA gene were designed on the basis of the symbiont gene sequences determined in this study. Specificity of the primers was also confirmed by homology searches against the public DNA databases. Diagnostic PCR was conducted under a temperature profile of 95 °C for 10 min, 40 cycles of 95 °C for 20 s, 62 °C for 20 s and 72 °C for 30 s, and 72 °C for 2 min. A DNA sample of *C. sikkimensis* from which *Curculioniphilus* infection had been confirmed (Toju *et al*., 2010) was used as positive control.

Molecular phylogenetic analysis
Multiple alignments of the nucleotide sequences were generated using the program MAFFT v6.813b (Katoh and Toh, 2008), followed by elimination of ambiguously aligned nucleotide sites using GBlocks Server v0.91b (Castresana, 2000). Best-fit substitution models for the aligned sequences were selected using the program Kakusan v4 (Tanabe, 2011). Maximum likelihood phylogenies were inferred using the software Treefinder (Jobb *et al.,* 2004) with the tool package Phylogears v1.5 (Tanabe, 2008), whereby parallelized tree search bootstrapping was conducted. Bayesian phylogenies were reconstructed using the program MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003).

Co-speciation analysis
The levels of topological congruence between the symbiont phylogenies and the host phylogenies were evaluated by a distance based, ParaFit analysis (Legendre *et al*., 2002) and a likelihood based, approximately unbiased test (Shimodaira, 2002). The distance matrices were calculated based on maximum likelihood trees using the wrapper program of ParaFit, CopyCat v1.00.14 (Meier-Kolthoff *et al*., 2007). Subsequently, a randomization test of congruence between the host and symbiont matrices were conducted (99 999 permutations). The approximately unbiased test compares the hypothesis of strict host–symbiont co-speciation to an alternative hypothesis allowing host shifts of symbionts. Under the hypothesis of strict co-speciation, a maximum likelihood phylogeny was reconstructed using Kakusan and Phylogears as described above after combining the sequence data of the hosts and the symbionts. The likelihood under this hypothesis was obtained by multiplying the likelihoods of all the nucleotide sites. Meanwhile, under the hypothesis allowing host shifts, a host phylogeny and a symbiont phylogeny were estimated independently; the likelihood under this hypothesis was obtained by multiplying the likelihoods of all the nucleotide sites of the hosts and the symbionts. The likelihoods under the two alternative hypotheses were compared based on the multi-scale bootstrapping of the log-likelihoods of respective nucleotide sites using the program CONSEL v0.1k with 10 sets of 10 000 bootstrap replicates (Shimodaira and Hasegawa, 2001).

Relative rate test
Relative rate tests were performed using the program RRTree (Robinson-Rechavi and Huchon, 2000) on the basis of 1257 unambiguously aligned nucleotide sites of 16S rRNA gene sequences with the maximum likelihood tree of Figure 1 used as a guide tree.

Accession numbers
The nucleotide sequences determined in this study were deposited in the DDBJ/EMBL/GenBank

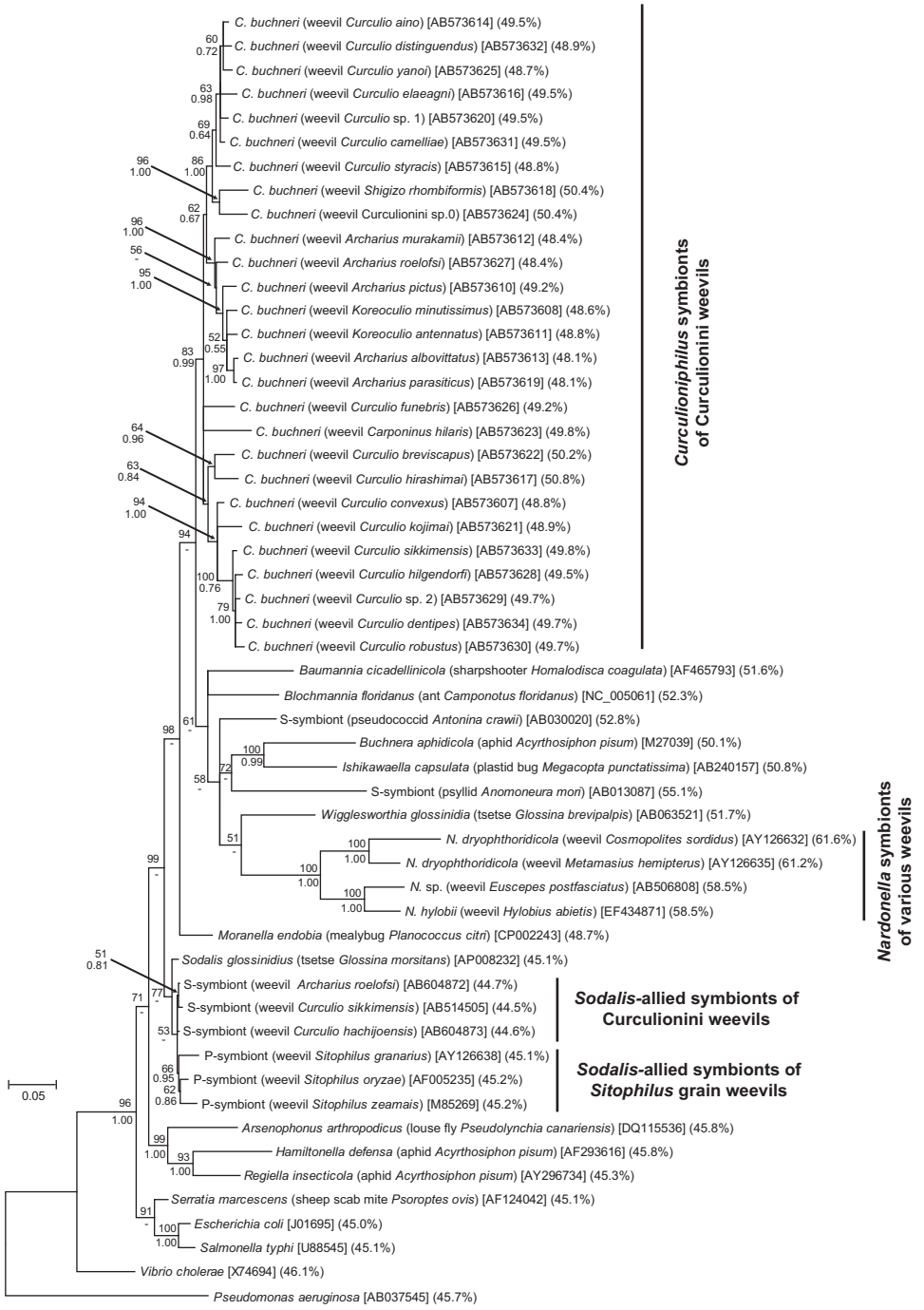

Figure 1 Molecular phylogenetic analysis of *Curculioniphilus* symbionts from Curculionini weevils on the basis of 16S rRNA gene sequences. A maximum likelihood tree inferred from 1257 unambiguously aligned nucleotide sites under the GTR + G model is shown. A Bayesian analysis under the GTR + G model yielded substantially the same result. Bootstrap values (> 50%; 100 replicates) of the maximum likelihood analysis and posterior probabilities (> 0.50) of the Bayesian analysis are shown above and below each node, respectively. *Sodalis*-allied symbionts identified from the Curculionini weevils and *Sitophilus* weevils, *Nardonella* symbionts of Dryophthorinae and Molytinae weevils, and other insect symbionts and free-living gammaproteobacteria are analyzed together. Sequence accession numbers and AT contents of the nucleotide sequences are shown in brackets and parentheses, respectively. For each insect symbiont, name of its host insect is indicated in parentheses. P-symbiont, primary symbiont; S-symbiont, secondary symbiont.

nucleotide sequence databases with accession numbers AB507712, AB573438–AB573634, AB604655–AB604677, AB604872–AB604873 and AB746368–AB746431 (for detail, see Supplementary Tables S1, S3 and S4).

Results

Cloning and sequencing of symbiont 16S rRNA genes from Curculionini weevils

In total, 36 species of Curculionini weevils were subjected to PCR, cloning and sequencing of

bacterial 16S rRNA gene, of which 27 species yielded 16S rRNA gene sequences highly similar to the sequences of *Curculioniphilus* symbionts of *Curculio sikkimensis*, *C. camelliae*, *C. dentipes* and *C. robustus* (Toju *et al*., 2010), and some of them also yielded 16S rRNA gene sequences of such facultative symbionts as *Sodalis*, *Wolbachia*, *Rickettsia* and *Spiroplasma*. Meanwhile, the *Curculioniphilus* sequences were not detected in the remaining nine species, *C. cerasorum*, *C. hachijoensis*, *C. koreanus*, *C. lateritius*, *C. maculanigra*, *C. morimotoi*, *C. ochrofasciatus*, *C. okumai* and *Archarius esakii* (Supplementary Table S1). We repeated PCR of the bacterial 16S rRNA gene for the nine species. No PCR amplification was observed for *A. esakii* and *C. cerasorum*, whereas the other seven species yielded the PCR products, which were cloned and sequenced. A variety of bacterial sequences derived from facultative symbionts such as *Sodalis*, *Wolbachia*, *Rickettsia*, *Spiroplasma* and *Lariskella*, and also those from putative gut associates/contaminants such as *Erwinia*, *Pseudomonas*, *Pantoea*, *Ochrobactorum*, *Enterobacter*, *Bradyrhizobium* and *Sphingomonas* were identified, but no *Curculioniphilus* sequences were obtained (Supplementary Table S3).

Besides the sequences of the primary symbiont *Curculioniphilus*, the following 16S rRNA gene sequences were identified from the Curculionini weevils: *Sodalis* from *C. hachijoensis* and *A. roelofsi* (Figure 1); *Wolbachia* from *C. hachijoensis*, *C. hilgendorfi*, *C. morimotoi*, *C. okumai*, *C.* sp. 2, *A. roelofsi* and *Koreoculio minutissimus* (Supplementary Figure S1A); *Rickettsia* from *C. aino*, *C. camelliae*, *C. elaeagni*, *C. hilgendorfi*, *C. kojimai*, *C. lateritius*, *C.* sp. 1, *A. pictus* and *K. minutissimus* (Supplementary Figure S1B); *Spiroplasma* from *A. albovittatus* (Supplementary Figure S1C); and *Lariskella* from *C. morimotoi* and *C. okumai* (Supplementary Figure S1D). These symbiont sequences neither formed a well-supported monophyletic group nor reflected the host systematics, as previously reported in *C. sikkimensis* (Toju and Fukatsu, 2011).

Diagnostic PCR of Curculioniphilus *symbionts*

Diagnostic PCR detection of the *Curculioniphilus* symbiont was positive for 27 weevil species and negative for remaining 9 species, which was in agreement with the 16S rRNA sequencing results (Supplementary Table S1). As for species with two or more specimens, no intraspecific variation in *Curculioniphilus* infection was identified (Supplementary Table S1). Most of the weevil species utilizing figs, fagaceous acorns and insect galls were associated with the *Curculioniphilus* symbionts (4 of 4, 6 of 6 and 7 of 9, respectively), whereas only 1 of 5 weevil species feeding on birches was infected with the symbiont (Supplementary Table S1; Figure 2).

Phylogenetic analysis of Curculioniphilus *symbionts*

Molecular phylogenetic analyses based on the 16S rRNA gene sequences revealed that the *Curculioniphilus* symbionts of the 27 Curculionini weevil species formed a clade in the *Gammaproteobacteria*. The *Curculioniphilus* clade was allied neither to *Nardonella* symbionts of diverse weevils nor to *Sodalis*-allied symbionts of *Sitophilus* weevils (Figure 1).

Phylogenetic analysis of Curculionini weevils

Figure 2 shows the molecular phylogeny based on sequences of two mitochondrial and three nuclear genes of the 36 Curculionini weevil species, which was largely concordant with previous systematic studies based on morphological characters (Morimoto, 1960, 1962; Hayashi *et al*., 1984). For example, species feeding on fagaceous acorns with relatively large bodies (6–10 mm in body length) formed a monophyletic group (clade A), so did species characterized by flat dorsal sides of elytra and cylindrical rostra (clade B). In addition, species characterized by small body size ($\sim$4 mm in body length) and insect gall feeding formed a monophyletic group (clade C). As for host plant usage, the following patterns were observed: (i) clade A members exclusively feed on fagaceous acorns; (ii) clade C members all live on insect galls; (iii) clade B members utilize various plant groups (birches, camellias, silverberries and others) as well as various plant parts (seeds, strobiles, flower buds and insect galls); and (iv) species feeding on figs constitute basal lineages.

Co-evolutionary analysis between Curculioniphilus *symbionts and their weevil hosts*

Figure 3 contrasts the phylogeny of the 27 Curculionini weevil species with the phylogeny of their *Curculioniphilus* symbionts. A distance-based co-speciation test showed that phylogenetic distances between operational taxonomic units were significantly congruent between the host phylogeny and the symbiont phylogeny (ParaFit method; $P = 0.00001$). The analysis also identified significant co-speciating patterns in 20 of the 27 operational taxonomic units examined (Figure 3). Furthermore, the strict co-speciation hypothesis was not rejected by a likelihood-based test ($P = 0.093$).

Phylogenetic analysis of Sodalis*-allied symbionts of weevils*

Figure 4 shows the phylogenetic relationship between *Sodalis*-allied symbionts from *Sitophilus* weevils (nine symbiont sequences representing three host species), some Curculionini weevils (eight symbiont sequences representing three host species), and other insects on the basis of their 16S rRNA gene sequences. The symbionts of *Sitophilus* weevils did not form a well-supported monophyletic clade, and the symbiont phylogeny did not

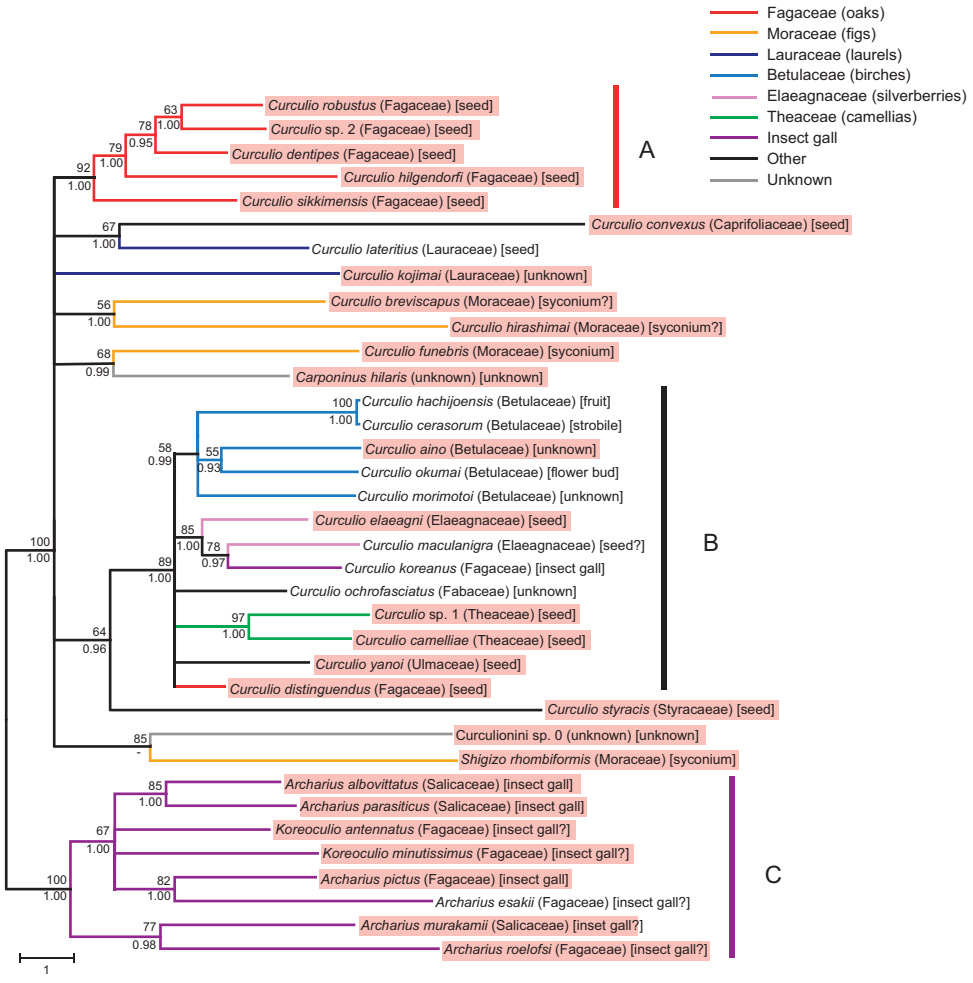

Figure 2 Molecular phylogenetic analysis of Curculionini weevils. A maximum likelihood tree inferred from 2686 unambiguously allied nucleotide sites of two mitochondrial (cytochrome oxidase subunit I (COI), 775-bp; cytochrome *b*, 382-bp) and three nuclear (elongation factor 1 α, 367-bp; 28S rRNA, 737-bp; phosphoglycerate mutase, 425-bp) genes is shown. A Bayesian analysis yielded substantially the same topology. Bootstrap values (> 50%; 100 replicates) of the maximum likelihood analysis and posterior probabilities (> 0.50) of the Bayesian analysis are shown above and below each node, respectively. See Supplementary Table S4 for sequence accession numbers and Supplementary Table S5 for substitution models. The clades A, B and C are indicated on the right side. For each of the weevil species, host plant family and tissue type are shown in parentheses and brackets, respectively. Evolutionary history of their host plants is inferred and mapped on the phylogeny by colored branches. Pink shades indicate association with the *Curculioniphilus* symbionts.

reflect the host systematics: the symbiont sequences from the same host species did not cluster but constituted distinct lineages in the phylogeny. Meanwhile, the symbionts of Curculionini weevils formed basal clusters in the phylogeny, and statistical supports for the groupings were generally low.

Co-evolutionary analysis between Sodalis*-allied symbionts and their* Sitophilus *weevil hosts*

Figure 5 contrasts the phylogeny of *Sitophilus* weevils with the phylogeny of their *Sodalis*-allied symbionts. No host–symbiont co-speciating pattern was recognizable in the weevil genus *Sitophilus*. A distance-based co-speciation test showed that phylogenetic distances between operational taxonomic units were incongruent between the host phylogeny and the symbiont phylogeny (ParaFit method; $P = 0.19$).

Molecular evolutionary aspects of Curculioniphilus *symbionts and* Sodalis*-allied symbionts of weevils*

The 16S rRNA gene sequences of the *Curculioniphilus* primary symbionts exhibited AT contents ranging from 48.1% to 50.8%, which were remarkably higher than the AT contents of free-living gammaproteobacteria around 45% (Figure 1). Meanwhile, the 16S rRNA gene sequences of the *Sodalis*-allied primary symbionts of *Sitophilus* weevils were around 45% in their AT contents, so were the sequences of the *Sodalis*-allied secondary symbionts of Curculionini weevils (Figure 4). The molecular evolutionary rate of the *Curculioniphilus* primary

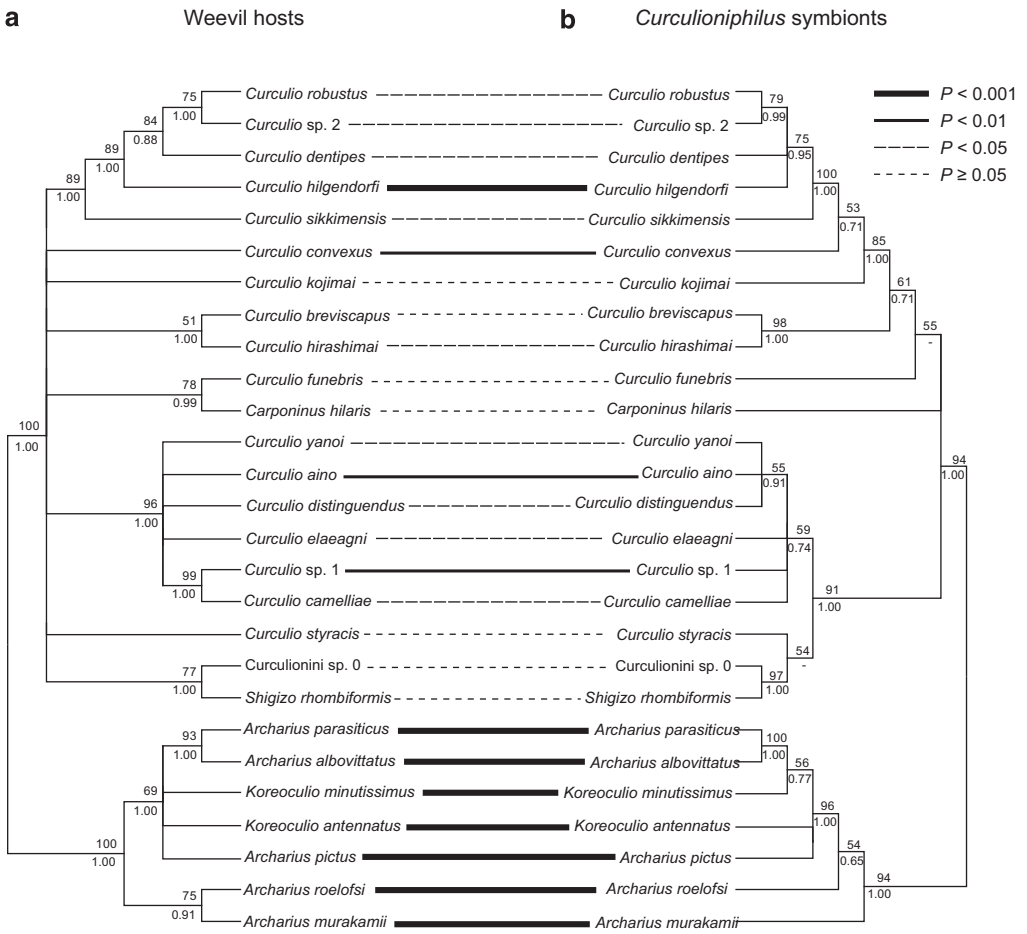

Figure 3 Co-speciation analysis between Curculionini weevil hosts and their *Curculioniphilus* symbionts. (**a**) A maximum likelihood phylogeny of 27 Curculionini weevil species inferred from 2689 unambiguously aligned nucleotide sites (cytochrome oxidase subunit I (COI), 775-bp; cytochrome *b* (Cyt*b*), 382-bp; elongation factor 1α (EF1α), 367-bp; 28S rRNA, 740-bp; phosphoglycerate mutase (Pglym), 425-bp). See Supplementary Table S5 for substitution models. (**b**) A maximum likelihood phylogeny of their *Curculioniphilus* symbionts inferred from 1458 unambiguously aligned nucleotide sites of 16S rRNA gene under the TVM + G model. In both trees, bootstrap values (> 50%; 100 replicates) of the maximum likelihood analysis and posterior probabilities (> 0.50) of Bayesian analysis are shown above and below each node, respectively. Host–symbiont associations are highlighted by lines between the trees, whose thickness indicates the significance of the association inferred by the ParaFit program (Legendre *et al.*, 2002).

symbionts was significantly higher than the evolutionary rates of the *Sodalis*-allied primary symbionts of *Sitiophilus* weevils ($P = 0.0047$), the *Sodalis*-allied secondary symbionts of Curculionini weevils ($P < 0.0001$), the tsetse secondary symbiont *Sodalis glossinidius* ($P < 0.0001$) and *Escherichia coli* ($P = 0.0293$; Table 1). The molecular evolutionary rate of the *Sodalis*-allied primary symbionts of *Sitophilus* weevils was significantly higher than the evolutionary rates of the *Sodalis*-allied secondary symbionts of Curculionini weevils ($P < 0.0001$) and the tsetse secondary symbiont *S. glossinidius* ($P = 0.0255$; Table 1).

Discussion

Curculioniphilus *as the primary symbiont co-speciating with Curculionini weevils*

We examined 36 weevil species of the tribe Curculionini, and identified 27 weevil species associated with *Curculioniphilus* (Supplementary Table S1). The phylogenetic relationship of the *Curculioniphilus* symbionts was congruent with the phylogenetic relationship of their host weevils (Figure 3). The host–symbiont co-speciation over evolutionary time must have been underpinned by stable vertical transmission of the bacteriocyte-associated symbiont through the host weevil generations via ovarial passage (Toju *et al.*, 2010). The AT-biased nucleotide compositions and the accelerated evolutionary rates of 16S rRNA gene of the *Curculioniphilus* symbionts are also suggestive of long-lasting host–symbiont co-evolution (Figure 1; Table 1). Based on these results, we conclude that *Curculioniphilus* comprises the primary symbiont clade associated with the Curculionini weevils. As *Nardonella* is the ancestral primary symbiont of weevils (Lefèvre *et al.*, 2004; Conord *et al.*, 2008), it is conjectured that *Curculioniphilus* was acquired by the common ancestor of the Curculionini weevils and took over the original symbiont.

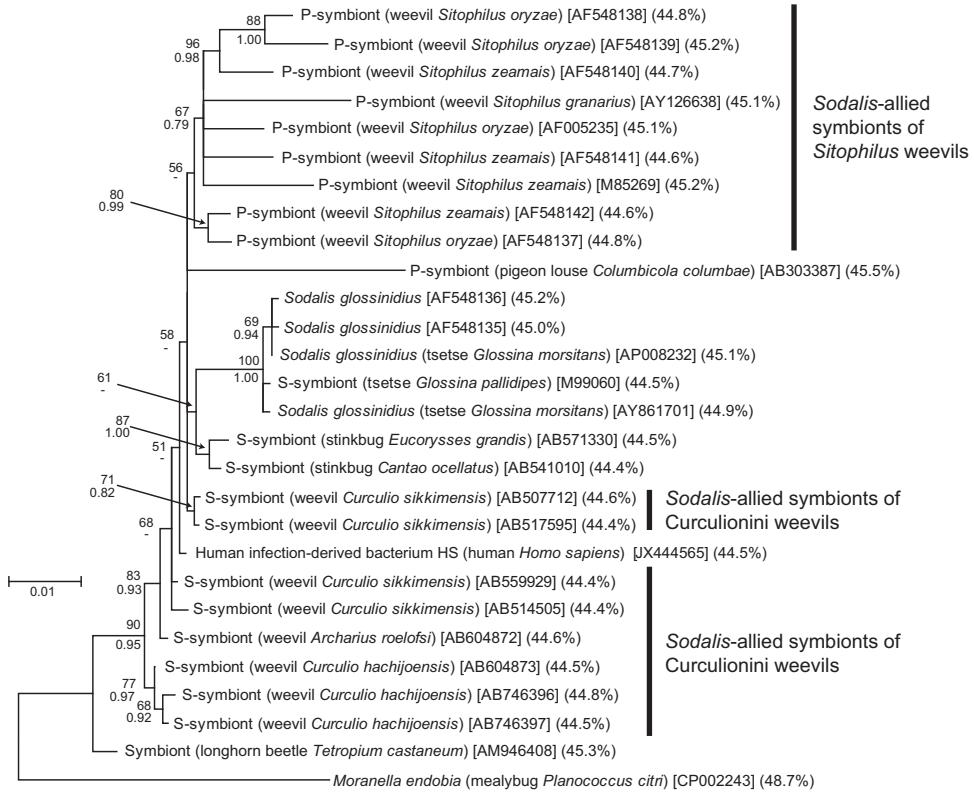

Figure 4 Molecular phylogenetic analysis of *Sodalis*-allied symbionts from Curculionini weevils, *Sitophilus* weevils, and various other insects on the basis of 16S rRNA gene sequences. A maximum likelihood tree inferred from 1278 unambiguously allied nucleotide sites under the J1 + G model is shown. A Bayesian analysis under the GTR + G model yielded substantially the same topology. Bootstrap values (> 50%; 100 replicates) of the maximum likelihood analysis and posterior probabilities (> 0.50) of the Bayesian analysis are shown above and below each node, respectively. Sequence accession numbers and AT contents of the nucleotide sequences are shown in brackets and parentheses, respectively. For each insect symbiont, name of its host insect is indicated in parentheses. P-symbiont, primary symbiont; S-symbiont, secondary symbiont.

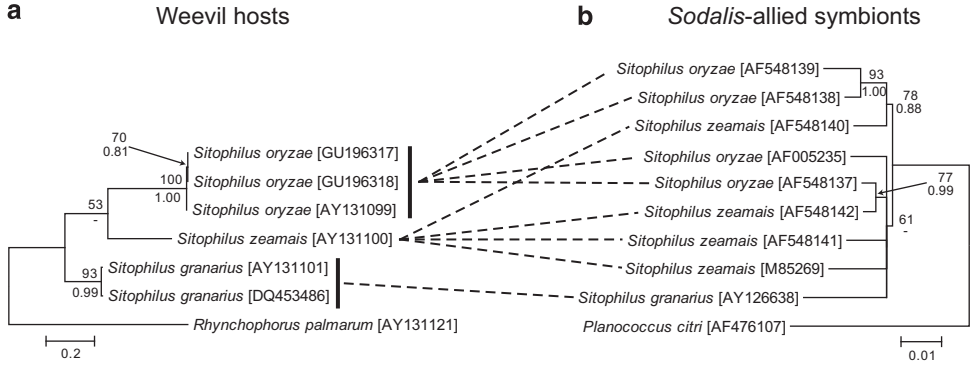

Figure 5 Phylogenetic comparison between *Sitophilus* weevil hosts and their *Sodalis*-allied symbionts. (**a**) A maximum likelihood phylogeny of six cytochrome oxidase subunit I (COI) gene sequences representing three *Sitophilus* species based on 467 unambiguously aligned nucleotide sites (J1 + G, F81 + G and TN93 + G models for the first, second and third codon positions, respectively). (**b**) A maximum likelihood phylogeny of nine 16S rRNA gene sequences from the three *Sitophilus* species based on 1281 unambiguously aligned nucleotide sites under the J1 + G model. In both trees, bootstrap values (> 50%; 100 replicates) of the maximum likelihood analysis and posterior probabilities (> 0.50) of Bayesian analysis are shown above and below each node, respectively. Host–symbiont relationships are indicated by dotted lines.

Occasional losses of Curculioniphilus *in the evolutionary course of Curculionini weevils*

Meanwhile, of 36 Curculionini weevil species we examined, 9 species are not associated with *Curculioniphilus* (Supplementary Table S1). The global host–symbiont co-speciation (Figure 3) and the local distribution of the *Curculioniphilus*-free species (Figure 2) indicate that *Curculioniphilus* has been occasionally lost in several host lineages independently, which highlights the dynamic evolutionary

Table 1 Relative-rate test for comparing the molecular evolutionary rates of 16S rRNA gene sequences between *Curculioniphilus* symbionts of Curculionini weevils, *Sodalis*-allied symbionts of *Sitophilus* grain weevils and their free-living relatives

Lineage 1	*Lineage 2*	*Outgroup*	*K1*[a]	*K2*[b]	*K1-K2*	*K1/K2*	P-*value*[c]
Curculioniphilus symbionts of Curculionini weevils[d]	*Escherichia coli* (J01695)	*Vibrio cholera* (X74694)	0.126	0.106	0.020	2.18	0.0293*
Sodalis-allied symbionts of *Sitophilus* weevils[e]	*Escherichia coli* (J01695)	*Vibrio cholera* (X74694)	0.121	0.107	0.014	1.63	0.1029
Sodalis-allied symbionts of Curculionini weevils[f]	*Escherichia coli* (J01695)	*Vibrio cholera* (X74694)	0.107	0.106	0.001	0.12	0.9061
Sodalis glossinidius (AP008232)	*Escherichia coli* (J01695)	*Vibrio cholera* (X74694)	0.107	0.106	0.001	0.14	0.8901
Curculioniphilus symbionts of Curculionini weevils[d]	*Sodalis*-allied symbionts of *Sitophilus* weevils[e]	*Escherichia coli* (J01695)	0.098	0.078	0.020	2.83	0.0047*
Curculioniphilus symbionts of Curculionini weevils[d]	*Sodalis*-allied symbionts of Curculionini weevils[f]	*Escherichia coli* (J01695)	0.099	0.063	0.036	5.14	< 0.0001*
Curculioniphilus symbionts of Curculionini weevils[d]	*Sodalis glossinidius* (AP008232)	*Escherichia coli* (J01695)	0.099	0.069	0.030	4.39	< 0.0001*
Sodalis-allied symbionts of *Sitophilus* weevils[e]	*Sodalis*-allied symbionts of Curculionini weevils[f]	*Escherichia coli* (J01695)	0.078	0.064	0.015	4.88	< 0.0001*
Sodalis-allied symbionts of *Sitophilus* weevils[e]	*Sodalis glossinidius* (AP008232)	*Escherichia coli* (J01695)	0.078	0.069	0.009	2.23	0.0255*
Sodalis-allied symbionts of Curculionini weevils[f]	*Sodalis glossinidius* (AP008232)	*Escherichia coli* (J01695)	0.063	0.069	–0.006	–1.60	0.1092

The maximum likelihood tree in figure 1 was used as a guide tree.
[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]*P*-value was generated using the program RRTree (Robinson-Rechavi and Huchon, 2000). Asterisks indicate statistically significant differences.
[d]*Curculioniphilus* symbionts of *C. sikkimensis* (AB573633), *A. pictus* (AB573610) and *K. antennatus* (AB573611).
[e]*Sodalis*-allied symbionts of *S. oryzae* (AF005235), *S. zeamais* (M85269) and *S. granarius* (AY126638).
[f]*Sodalis*-allied symbionts of *C. sikkimensis* (AB514505), *A. roelofsi* (AB604872) and *C. hachijoensis* (AB604873).

history of the endosymbiosis among the Curculionini weevils. For these *Curculioniphilus*-free species, whether the symbionts were simply lost or replaced by unidentified microbial associates deserves further analyses.

Evolutionary dynamics of Curculioniphilus *among Curculionini weevils: possible relevance to biological roles of the symbiont and ecological aspects of the host*

The evolutionary stability and the co-speciating pattern in the host–symbiont association (Figure 3) favor the hypothesis that *Curculioniphilus* has some biological roles for the host weevils, such as provisioning of nutritional components deficient in their food plants and/or detoxification of plant defense chemicals (Toju *et al*., 2010; Toju and Fukatsu, 2011). Meanwhile, considering the pattern of repeated evolutionary losses of *Curculioniphilus* (Figure 2), the necessity of the symbiont for the host weevils might be not stringent but could be mitigated/circumvented under certain ecological/environmental conditions. A candidate ecological factor relevant to the symbiont losses may be host plant switches in the Curculionini (see Figure 2), but the causal relationship is currently elusive and deserves more detailed studies. Notably, a recent study reported that, in the weevil *Euscepes postfasciatus*, even the ancient primary symbiont *Nardonella* is beneficial but not essential for the host: symbiont-free weevils were able to grow and reproduce, although they suffered retarded growth, smaller body size and reduced fecundity in comparison with normal symbiotic weevils (Kuriwada *et al*., 2010). Based on these evolutionary patterns and circumstances, we suggest that *Curculioniphilus* is certainly the bacteriocyte-associated primary symbiont of beneficial nature in the majority of Curculionini weevils, but may be not necessarily essential for the host insects under particular ecological conditions, which might have prompted the occasional losses of the symbiont in some host lineages.

Secondary symbionts of Curculionini weevils

A previous study reported that, in addition to the primary symbiont *Curculioniphilus*, several secondary symbionts including *Wolbachia*, *Rickettsia*, *Spiroplasma* and *Sodalis* are prevalent in natural populations of *C. sikkimensis* (Toju and Fukatsu, 2011). In this study, we demonstrated that diverse Curculionini weevils are also associated with these facultative secondary symbionts (Figure 4; Supplementary Figure S1A–C). In addition, from *C. morimotoi* and *C. okumai*, we detected *Lariskella* symbionts (Supplementary Figure S1D), which belong to a recently described facultative symbiont clade associated with stinkbugs, fleas and ticks (Matsuura *et al*., 2012). The finding that *Sodalis*-allied facultative symbionts are present in natural Curculionini populations is evolutionarily interesting, shedding light on the origin of the *Sodalis*-allied primary symbionts of *Sitophilus* weevils, as discussed later.

Sodalis-*allied primary symbiont of* Sitophilus *weevils: lack of host–symbiont co-speciation*

The weevil genus *Sitophilus*, consisting of 14 described species, is known for the notorious pest species *S. oryzae*, *S. zeamais* and *S. granarius* that infest stored crop products such as rice, wheat and maize (Plarre, 2010). The *Sodalis*-allied primary symbionts of the *Sitophilus* weevils, often referred to by the acronym SOPE or SPE (after *Sitophilus* (*oryzae*) primary endosymbiont), are localized in the midgut-associated bacteriome and also in the female ovaries, vertically transmitted through host generations via ovarial passage, and beneficial for host growth and reproduction (Heddi and Nardon, 2005). As most of the other Dryophthorinae weevils harbor the ancient primary symbiont *Nardonella* (Lefèvre *et al.*, 2004), it has been suggested that the *Sodalis*-allied symbiont was acquired by an ancestor of the *Sitophilus* weevils, replaced the original symbiont *Nardonella*, and established as a new primary symbiont (Lefèvre *et al.*, 2004; Heddi and Nardon, 2005). The symbiont replacement process in the *Sitophilus* weevils looks quite similar to that in the Curculionini weevils, but subsequent host–symbiont co-evolutionary trajectories are strikingly different between them: unlike *Curculioniphilus*, the *Sodalis*-allied primary symbionts exhibited no host–symbiont co-speciation (Figure 5), no bias in nucleotide compositions (Figure 1), and less accelerated evolutionary rates (Table 1) in the *Sitophilus* weevils. On account of the small number of *Sitophilus* weevil species examined and the relatively low statistical supports for the phylogeny of *Sodalis*-allied symbionts, the host–symbiont phylogenetic incongruence may be not robust statistically, but it is evidently different from the remarkable host–symbiont co-speciating pattern in the Curculionini weevils.

Evolutionary dynamics of Sodalis-*allied symbiont in* Sitophilus *weevils: possible evolutionary processes underlying the promiscuous host–symbiont relationship*

These evolutionary patterns suggest that the *Sodalis*-allied symbiont of *Sitophilus* weevils is, although bacteriocyte-associated and beneficial (Heddi and Nardon, 2005), similar to facultative secondary symbionts like *Wolbachia* in diverse insects and *Serratia*, *Hamiltonella* and *Regiella* in aphids, at least in some aspects (Werren *et al.*, 2008; Oliver *et al.*, 2010). This finding is striking in that a number of bacteriocyte-associated primary symbionts of beneficial nature, including *Buchnera, Wigglesworthia* and others, are generally co-speciating with their host insects over evolutionary time (Baumann, 2005; Moran *et al.*, 2008). In theory, the promiscuous host–symbiont relationship in the *Sitophilus* weevils can be generated by either of the following processes: (i) horizontal transfers of the symbiont across different host lineages within the genus *Sitophilus*; (ii) horizontal acquisitions of the symbiont from outside, possibly from different insect sources; or (iii) repeated acquisitions from free-living symbiont pool in the environment, as reported in some stinkbugs (Kikuchi *et al.*, 2007, 2011). Hereafter, we discuss these possibilities under the light of phylogenetic, evolutionary and other lines of evidence available to date.

Given that the scenario (i) applies, symbiont swapping within and between the host lineages would result in occurrences of genetically identical or close symbiont genotypes within and between the host insect species, which would generate a compact symbiont phylogeny with short terminal branches, as commonly observed with *Wolbachia*, *Rickettsia*, *Serratia*, *Hamiltonella*, *Regiella* and other facultative insect symbionts (Russell *et al.*, 2003; Baldo *et al.*, 2006; Weinert *et al.*, 2009). Oddly, however, such phylogenetic patterns are not observed with the *Sodalis*-allied *Sitophilus* symbionts: the symbiont genotypes are remarkably divergent within and between the host weevil species, with long terminal branches giving a comb- or star-like appearance of the phylogeny (Figures 4 and 5). Hence, we suppose that the scenario (i) does not fit well to the observed data.

If the scenario (ii) applies, the *Sodalis*-allied primary symbionts of *Sitophilus* weevils must have been occasionally acquired from somewhere via repeated horizontal transfers, and the candidate sources are the *Sodalis*-allied facultative symbionts of other insects. For a long time, *Sodalis glossinidius* and allied insect symbionts have been known only from *Glossina* tsetse flies and *Sitophilus* weevils (Dale and Maudlin, 1999; Heddi *et al.*, 1999). However, recent studies unveiled universal occurrences of *Sodalis*-allied symbionts in diverse insects such as bird lice (Fukatsu *et al.*, 2007), louse flies (Nováková and Hypša, 2007; Chrudimský *et al.*, 2012), weevils (Toju and Fukatsu, 2011), longicorn beetles (Grünwald *et al.*, 2010) and stinkbugs (Kaiwa *et al.*, 2010, 2011). In this study, notably, we demonstrated that *Sodalis*-allied facultative symbionts are commonly present in natural populations of Curculionini weevils, which constitute basal lineages in the phylogeny of *Sodalis*-allied symbionts of various insects including *Sitophilus* weevils (Figure 4). Therefore, it seems plausible, although speculative, that the weevil-associated *Sodalis*-allied facultative bacteria might have served as the evolutionary sources for the *Sodalis*-allied primary symbionts of the *Sitophilus* weevils. To confirm this hypothesis, future surveys should focus on whether *Sitophilus* and other Dryophthorinae weevils are associated with *Sodalis*-allied symbionts of facultative type.

In the context of the scenario (iii), the recent finding of a *Sodalis*-allied cultivable bacterium from human wound (Clayton *et al.*, 2012) is of interest. Meaningfully, the human-derived *Sodalis*-allied

bacterium was placed in the basal position of the phylogeny as *Sodalis*-allied symbionts of Curculionini weevils were (Figure 4). It should be noted that the tsetse-associated symbiont *S. glossinidius* can be cultured in cell-free media (Dale and Maudlin, 1999), suggesting the possibility of free-living lifestyle of some *Sodalis*-allied bacterial strains, which may potentially serve as environmental pool for symbiont acquisition.

Repeated acquisitions and replacements of Sodalis*-allied symbionts in* Sitophilus *weevils: putative factors relevant to the symbiont evolutionary dynamics*

In summary, the host–symbiont phylogenetic incongruence in the *Sitophilus* weevils may be best accounted for by repeated horizontal acquisitions and replacements of their *Sodalis*-allied primary symbionts, whose origins are weevil- or other insect-associated *Sodalis*-allied facultative symbionts, or free-living *Sodalis*-allied bacteria in the environment. The host–symbiont promiscuity might have been facilitated by the 'beneficial but not essential' nature of the symbiotic association. Previous studies reported that *Sitophilus* weevil strains experimentally deprived of the *Sodalis*-allied symbiont could be established and continuously maintained (Nardon, 1973), but these strains exhibited paler body color, soft cuticle, slower growth, reduced fecundity, and lower flight activity in comparison with normal symbiotic strains (King and Sang, 1959; Grenier *et al.*, 1986, 1994; Nardon and Nardon, 1998). Also host plant difference might have affected the process of symbiont replacements. Lefèvre *et al.* (2004) reported that, distinct from *S. oryzae*, *S. zeamais* and *S. granarius* that live on monocot grain seeds, *S. linealis* feeding on dicot tamarind seeds was aposymbiotic, and argued the possibility of symbiont loss associated with the host plant shift.

Conclusion and perspective

In this study, we highlighted two remarkable evolutionary events in weevils, namely independent replacements of the ancient bacteriocyte-associated primary symbiont *Nardonella* by different microorganisms, *Curculioniphilus* symbiont in the tribe Curculionini and *Sodalis*-allied symbiont in the genus *Sitophilus*. Despite replacing the same original symbiont, these two lineages of newcomer symbionts exhibited strikingly different evolutionary patterns: host–symbiont co-speciation in the *Curculiophilus*–Curculionini association vs host–symbiont promiscuity in the *Sodalis*–*Sitophilus* association. In both weevil lineages, further symbiont losses have occurred occasionally, which might be relevant to food plant switching of the host weevils. The evolutionary origins of the *Sodalis*-allied primary symbiont in *Sitophilus* weevils might be, although speculative, either *Sodalis*-allied facultative symbionts associated with diverse weevils or free-living *Sodalis*-allied bacteria in the environment, whereas the evolutionary origin of the *Curculioniphilus* symbiont in Curculionini weevils is still elusive.

In describing the diversity of various host–symbiont relationships, we tend to use dichotomous categorizations for simplicity, such as primary symbiont vs secondary symbiont, obligate symbiont vs facultative symbiont, beneficial symbiont vs parasitic symbiont, co-speciating symbiont vs promiscuous symbiont, and so on. However, the real world is usually better described by continuity spanning the extremes, which is impressively illustrated by the endosymbiont diversity among the weevils.

Acknowledgements

We thank, H Fujimoto, H Hirano, K Kume, H Makihara, K Matsushita and H Yoshitake for their help in collecting insect specimens and H Kojima and K Morimoto for providing ecological information of Curculionini weevils. This work was funded by the Japan Society for the Promotion of Science (No. 2004352) to HT and by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN) to TF.

References

Alonso-Zarazaga MA, Lyal CHC. (1999). *A World Catalogue of Families and Genera of Curculionoidea (Insecta: Coleoptera)*. Entomopraxis: Barcelona, Spain.

Baldo L, Dunning Hotopp JC, Jolley KA, Bordenstein SR, Biber SA, Choudhury RR *et al.* (2006). Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Appl Env Microbiol* **72**: 7098–7110.

Baumann P. (2005). Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu Rev Microbiol* **59**: 155–189.

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

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

Castresana J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* **17**: 540–552.

Chrudimský T, Husník F, Nováková E, Hypša V. (2012). *Candidatus* Sodalis melophagi sp. nov.: phylogenetically independent comparative model to the tsetse fly symbiont *Sodalis glossinidius*. *PLoS One* **7**: e40354.

Clayton AL, Oakeson KF, Gutin M, Pontes A, Dunn DM, von Niederhausern AC *et al.* (2012). A novel human-infection-derived bacterium provides insights into the evolutionary origins of mutualistic insect–bacterial symbioses. *PLoS Genet* **8**: e1002990.

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 Evol Microbiol* **49**: 267–275.

Farrell BD. (1998). 'Inordinate fondness' explained: why are there so many beetles? *Science* **281**: 555–559.

Fujimoto H. (2004). The list of Japanese Curculioninae. *Newsletter Japanese Weevil Information Network* **3**: 2–6. [In Japanese].

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

Fukatsu T, Aoki S, Kurosu U, Ishikawa H. (1994). Phylogeny of Cerataphidini aphids revealed by their symbiotic microorganisms and basic structure of their galls: implications for host-symbiont coevolution and evolution of sterile soldier castes. *Zool Sci* **11**: 613–623.

Fukatsu T, Ishikawa H. (1992). A novel eukaryotic extracellular symbiont in an aphid, *Astegopteryx styraci* (Homoptera, Aphididae, Hormaphidinae). *J Insect Physiol* **38**: 765–773.

Fukatsu T, Ishikawa H. (1996). Phylogenetic position of yeast-like symbiont of *Hamiltonaphis styraci* (Homoptera, Aphididae) based on 18S rDNA sequence. *Insect Biochem Mol Biol* **26**: 383–388.

Fukatsu T, Koga R, Smith WA, Tanaka K, Nikoh N, Sasaki-Fukatsu K *et al.* (2007). Bacterial endosymbiont of the slender pigeon louse, *Columbicola columbae*, allied to endosymbionts of grain weevils and tsetse flies. *Appl Env Microbiol* **73**: 6660–6668.

Fukatsu T, Nikoh N. (1998). Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). *Appl Env Microbiol* **64**: 3599–3606.

Grenier AM, Nardon C, Nardon P. (1994). The role of symbiotes in flight activity of *Sitophilus* weevils. *Entomol Exp Appl* **70**: 201–208.

Grenier AM, Nardon P, Bonnot G. (1986). Importance de la symbiose dans la croissance des populations de Sitophilus oryzae L. (Coleoptère Curculionidae). Etude théorique et expérimentale. *Acta oecologica—Oecologia Applicata* **7**: 93–110.

Grünwald S, Pilhofer M, Höll W. (2010). Microbial associations in gut systems of wood- and bark-inhabiting longhorned beetles [Coleoptera: Cerambycidae]. *Syst Appl Microbiol* **33**: 25–34.

Hayashi K, Morimoto K, Kimoto S. (1984). *The Coleoptera of Japan in Color Vol. IV*. Hoikusha Publishing Co., Ltd: Osaka, Japan [In Japanese].

Heddi A, Grenier AM, Khatchadourian C, Charles H, Nardon P. (1999). Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. *Proc Natl Acad Sci USA* **96**: 6814–6819.

Heddi A, Nardon P. (2005). *Sitophilus oryzae* L.: a model for intracellular symbiosis in the Dryophthoridae weevils (Coleoptera). *Symbiosis* **39**: 1–11.

Hongoh Y, Ishikawa H. (2000). Evolutionary studies on uricases of fungal endosymbionts of aphids and planthoppers. *J Mol Evol* **51**: 265–277.

Hosokawa T, Fukatsu T. (2010). *Nardonella* endosymbiont in the West Indian sweet potato weevil *Euscepes postfasciatus* (Coleoptera: Curculionidae). *Appl Entomol Zool* **45**: 115–120.

Hughes J, Vogler AP. (2004a). Ecomorphological adaptation of acorn weevils to their oviposition site. *Evolution* **58**: 1971–1983.

Hughes J, Vogler AP. (2004b). The phylogeny of acorn weevils (genus *Curculio*) from mitochondrial and nuclear DNA sequences: the problem of incomplete data. *Mol Phylogenet Evol* **32**: 601–615.

Jobb G, Von Haeseler A, Strimmer K. (2004). TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evol Biol* **4**: 18.

Kaiwa N, Hosokawa T, Kikuchi Y, Nikoh N, Meng XY, Kimura N *et al.* (2010). Primary gut symbiont and secondary, *Sodalis*-allied symbiont of the scutellerid stinkbug *Cantao ocellatus*. *Appl Env Microbiol* **76**: 3486–3494.

Kaiwa N, Hosokawa T, Kikuchi Y, Nikoh N, Meng XY, Kimura N *et al.* (2011). Bacterial symbionts of the giant jewel stinkbug *Eucoryssus grandis* (Hemiptera: Scutelleridae). *Zool Sci* **28**: 169–174.

Katoh K, Toh H. (2008). Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinformatics* **9**: 286–298.

Kikuchi Y, Hosokawa T, Fukatsu T. (2007). Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial symbiont from the environment every generation. *Appl Env Microbiol* **73**: 4308–4316.

Kikuchi Y, Hosokawa T, Fukatsu T. (2011). An ancient but promiscuous host-symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts. *ISME J* **5**: 446–460.

King RC, Sang JH. (1959). Oögenesis in adult *Drosophila melanogaster*. VIII. The role of folic acid in oögenesis. *Growth* **23**: 37–53.

Kuriwada T, Hosokawa T, Kumano N, Shiromoto K, Haraguchi D, Fukatsu T. (2010). Biological role of *Nardonella* endosymbiont in its weevil host. *PLoS ONE* **5**: 165–190.

Lefèvre C, Charles H, Vallier A, Delobel B, Farrell B, Heddi A. (2004). Endosymbiont phylogenesis in the Dryophthoridae weevils: evidence for bacterial replacement. *Mol Biol Evol* **21**: 965–973.

Legendre P, Desdevises Y, Bazin E. (2002). A statistical test for host-parasite coevolution. *Syst Biol* **51**: 217–234.

Matsuura Y, Kikuchi Y, Meng XY, Koga R, Fukatsu T. (2012). Novel clade of alphaproteobacterial endosymbionts associated with stinkbugs and other arthropods. *Appl Env Microbiol* **78**: 4149–4156.

McKenna DD, Sequeira AS, Marvaldi AE, Farrell BD. (2009). Temporal lags and overlap in the diversification of weevils and flowering plants. *Proc Natl Acad Sci USA* **106**: 7083–7088.

Meier-Kolthoff JP, Auch AF, Huson DH, Göker M. (2007). CopyCat: cophylogenetic analysis tool. *Bioinformatics* **23**: 898–900.

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

Morimoto K. (1960). Revision of the subfamily Curculioninae from Japan I (Coleoptera). *Mushi* **33**: 89–104.

Morimoto K. (1962). Revision of the subfamily Curculioninae from Japan II (Coleoptera). *Mushi* **36**: 21–40.

Nardon P. (1973). Obtention d'une souche aposymbiotique chez le charancon Sitophilus sasakii Tak: differentes methods et comparaison avec la souche symbiotique d'origine. *CR Acad Sci* **277**: 981–984.

Nardon P, Nardon C. (1998). Morphology and cytology of symbiosis in insects. *Ann Soc Entomol Fr* **34**: 105–134.

Nováková E, Hypša V. (2007). A new *Sodalis* lineage from bloodsucking fly *Craterina melbae* (Diptera, Hippoboscoidea) originated independently of the tsetse flies

symbiont *Sodalis glossinidius*. *FEMS Microbiol Let* **269**: 131–135.
Oliver KM, Degnan PH, Burke GR, Moran NA. (2010). Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol* **55**: 247–266.
Plarre R. (2010). An attempt to reconstruct the natural and cultural history of the granary weevil, *Sitophilus granarius* (Coleoptera: Curculionidae). *Eur J Entomol* **107**: 1–11.
Robinson-Rechavi M, Huchon D. (2000). RRTree: relative-rate tests between groups of sequences on a phylogenetic tree. *Bioinformatics* **16**: 296–297.
Ronquist F, Huelsenbeck JP. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.
Russell JA, Latorre A, Sabater-Muñoz B, Moya A, Moran NA. (2003). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Mol Ecol* **12**: 1061–1075.
Shimodaira H. (2002). An approximately unbiased test of phylogenetic tree selection. *Syst Biol* **51**: 492–508.
Shimodaira H, Hasegawa M. (2001). CONSEL: for assessing the confidence of phylogenetic tree selection. *Bioinformatics* **17**: 1246–1247.
Stork NE. (2003). Biodiversity. In: Resh VH, Carde RT (eds) *Encyclopedia of Insects*. Academic Press: San Diego, CA, USA, pp 85–91.
Sugiura S, Yamazaki K, Hishi T. (2002). A cecidophagous weevil, *Curculio albovittatus* (Coleoptera: Curculionidae), in the gall of *Pontania* sp. (Hymenoptera: Tenthredinidae). *Entomol Sci* **5**: 193–196.
Tanabe AS. (2008). 'Phylogears version 1.5', software distributed by the author at http://www.fifthdimension.jp/.
Tanabe AS. (2011). Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional, and separate models for combined molecular phylogenetic analyses of multilocus sequence data. *Mol Ecol Notes* **11**: 914–921.
Toju H, Fukatsu T. (2011). Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. *Mol Ecol* **20**: 853–868.
Toju H, Hosokawa T, Koga R, Nikoh N, Meng XY, Kimura N *et al.* (2010). '*Candidatus* Curculioniphilus buchneri,' a novel clade of bacterial endocellular symbionts from weevils of the genus *Curculio*. *Appl Env Microbiol* **76**: 275–282.
Weinert LA, Werren JH, Aebi A, Stone GN, Jiggins FM. (2009). Evolution and diversity of *Rickettsia* bacteria. *BMC Biol* **7**: 6.
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.

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