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ORIGINAL RESEARCH PAPER

Fungal and bacterial endosymbionts of eared leafhoppers of the subfamily Ledrinae (Hemiptera: Cicadellidae)

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Abstract In general, leafhoppers (Hemiptera: Cicadellidae) possess a pair of bacteriomes in the abdomen, which harbor two types of obligate bacterial symbionts: a very ancient *Sulcia* symbiont and another ancient *Nasuia*-allied co-symbiont (or a younger *Baumannia* co-symbiont). However, when we inspected three eared leafhoppers of the subfamily Ledrinae, namely *Ledra auditura* Walker, *Ledropsis discolor* (Uhler) and *Tituria angulata* (Matsumura), *L. discolor* harbored only *Sulcia* symbiont while *L. auditura* and *T. angulata* possessed no bacterial symbionts. Instead, all the species possessed specialized cells full of yeast-like fungal symbionts within fat bodies. Molecular phylogenetic analysis revealed that the fungal symbionts are placed within the entomoparasitic fungal genus *Ophiocordyceps* (Ascomycota: Hypocreales: Ophiocordycipitaceae). These results suggest the possibility that (1) the fungal symbiont was acquired in the evolutionary course of the Ledrinae, (2) the original fungus was likely an entomoparasite of the genus *Ophiocordyceps*, (3) the fungal symbiont replaced the *Nasuia*-allied symbiont in an ancestral lineage, and (4) even the ancient *Sulcia* symbiont was finally lost and taken over by the fungal symbiont. Meanwhile, the possibility of multiple independent fungal acquisitions from closely related entomoparasitic *Ophiocordyceps* fungi cannot be excluded. Our finding uncovers an evolutionary process from a prokaryotic essential symbiosis to a eukaryotic one.

Keywords Ledrinae · Leafhopper · Symbiont · *Ophiocordyceps* · *Sulcia*

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Introduction

Diverse insects and other organisms are associated with symbiotic microbes, which entail a variety of ecological and evolutionary consequences (Bourtzis and Miller 2003; Buchner 1965; Moran et al. 2008; Oliver et al. 2010; Werren et al. 2008). In particular, plant-sucking insects of the order Hemiptera are highly dependent on their microbial partners and possess well-developed symbiotic systems in their specialized tissues, cells or gut regions. Since plant sap is usually deficient in proteins, vitamins and other nutrients, the insects require symbiont-provisioned essential amino acids, B vitamins and other nutrients for their growth and survival (Baumann 2005; Douglas 1998; Kikuchi 2009; Moran et al. 2008; Salem et al. 2015).

Leafhoppers (Hemiptera: Auchenorrhyncha: Cicadomorpha: Cicadellidae) consist of over 20,000 described species in the world (Forero 2008), whose growth and survival are dependent on symbiotic microorganisms (Douglas 1988; Noda et al. 2012). In general, leafhoppers possess a pair of symbiotic organs, called bacteriomes, in the abdomen, which harbor two types of obligate bacterial symbionts: a very ancient and highly conserved flavobacterial *Sulcia* symbiont originating from the common ancestor of the

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Auchenorrhyncha (Moran et al. 2005) and another ancient betaproteobacterial *Nasuia*-allied co-symbiont presumably dating back to the common ancestor of the Cicadomorpha (Bennett and Moran 2013; Koga et al. 2013; Noda et al. 2012). On the other hand, some leafhopper lineages exhibit different symbiotic microbial associates. For example, in the bacteriomes of leafhopper species of the tribe Proconiini, so-called sharpshooters, their bacteriomes harbor *Sulcia* and a gammaproteobacterial *Baumannia* symbiont (Moran et al. 2003; Takiya et al. 2006), suggesting replacement of the co-symbiont from *Nasuia*-allied to *Baumannia* in the lineage of sharpshooters (Bennett and Moran 2013, 2015). Genomics of *Sulcia*, *Nasuia* and *Baumannia* suggested their cooperative provisioning of essential amino acids and other nutrients deficient in the plant sap diet of the host leafhoppers (Bennett and Moran 2013; Bennett et al. 2014, 2016; McCutcheon and Moran 2007; Wu et al. 2006). On the other hand, *Sulcia* and a gammaproteobacterial *Arsenophonus* symbiont were present in *Macrosteles laevis* (Ribaut) (Kobialka et al. 2016), whereas *Sulcia* and a gammaproteobacterial *Sodalis* symbiont were found in *Cicadella viridis* (Linnaeus) (Michalik et al. 2014), probably representing independent symbiont-replacing events. In some leafhoppers such as *Scaphoideus titanus* Ball, strikingly, neither *Sulcia*, *Nasuia* nor *Baumannia* was found, but a yeast-like fungal symbiont and a facultative symbiont *Cardinium* were detected (Sacchi et al. 2008). These observations highlight repeated acquisitions, losses, replacements and diversification of symbiotic microorganisms in the evolutionary history of the leafhoppers (Bennett and Moran 2013; Bennett et al. 2016).

The subfamily Ledrinae is a small group of leafhoppers consisting of some 38 genera and 300 species in the world (Jones and Deitz 2009). Most members of the Ledrinae have conspicuous ear-like projections on their head and/or thorax, after which they are referred to as eared leafhoppers. Notably, an early histological study reported that a European ledrine species, *Ledra aurita* (Linnaeus), possesses no bacterial symbiont but a yeast-like fungal symbiont (Buchner 1925), although no further study has been conducted.

Entomoparasitic fungi of the genus *Cordyceps*, commonly known as Touchu-Kasou in Japanese, Dong Chong Xia Cao in Chinese, and Plant Worms or Vegetable Wasps in English, embrace over 400 described species in the world,

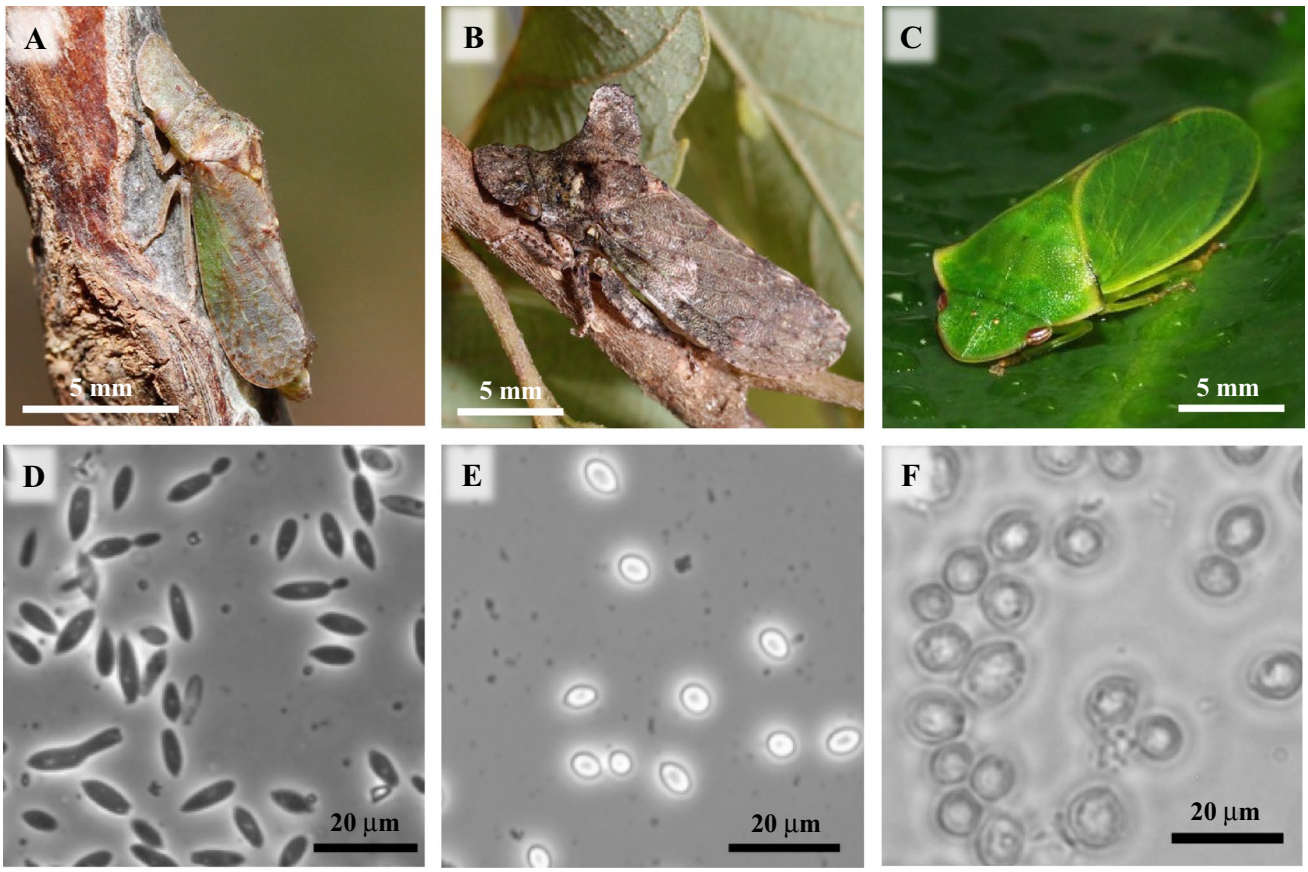


Fig. 1 Eared leafhoppers and their fungal symbionts. Adult insects of **a** *Ledropsis discolor,* **b** *Ledra auditura* and **c** *Tituria amgulata*. Phase contrast micrographs of fungal symbionts isolated from **d** *L. discolor*, **e** *L. auditura* and **f** *T. angulata*

and some species are famous for their potent use in Chinese medicine (Kobayashi 1982; Mains 1958; Shimizu 1994). A recent systematic revision based on molecular phylogenetic data divided the conventional *Cordyceps* into several genera including *Cordyceps*, *Elaphocordyceps* and *Ophiocordyceps* (Sung et al. 2007). Previous studies showed that some yeast-like symbionts of aphids and planthoppers are phylogenetically allied to *Cordyceps/Ophiocordyceps* fungi (Fukatsu and Ishikawa 1996; Noda et al. 1995; Suh et al. 2001).

In this study, we investigated the endosymbiotic microorganisms of three eared leafhopper species, *Ledropsis discolor* (Uhler) (Fig. 1a), *Ledra auditura* Walker (Fig. 1b) and *Tituria angulata* (Matsumura) (Fig. 1c). Our results uncovered an evolutionary transition from the ancestral bacterial co-symbiosis through a bacterium-fungus dual symbiosis to a fungus-only symbiosis in the evolutionary course of the ledrine leafhoppers, where the fungal symbionts were derived from entomoparasitic fungi of the genus *Ophiocordyceps*.

Materials and methods

Insects

Samples of the eared leafhoppers used in this study are listed in Table 1. The insects were collected by either light trapping or tree sweeping and preserved in acetone (Fukatsu 1999). Most of the samples were adult insects, whose abdomen was detached and cut into halves in the midline, and a half was subjected to DNA analysis while another half was used for histological analysis. In addition, we obtained a *L. discolor* nymph and a *T. angulata* nymph, which were used for detailed histological analysis (see Table 1).

DNA sequencing

Each dissected abdominal sample was placed in a 1.5-ml plastic tube, homogenized with a plastic pestle and subjected to DNA extraction using the QIAamp DNA mini kit (QIAGEN). For *L. discolor* samples, freeze-thawing and mixing with fine glass beads were also attempted to facilitate disruption of the fungal cell walls. A 1.5-kb region of the fungal 18S rRNA gene was amplified by PCR with primers NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) (White et al. 1990) and FS2 (5′- TAG GNA TTC CTC GTT GAA GA-3′) (Nikoh and Fukatsu 2000) under a temperature profile of 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 5 min. A 1.5-kb region of the 16S rRNA gene of flavobacteria, including *Sulcia* symbionts of diverse hemipteran insects, was amplified by PCR with primers 10CFBFF (5′-AGA GTT

Table 1 Samples of eared leafhoppers of the subfamily Ledrinae examined in this study with nucleotide sequence accession numbers

Insect species	Collection locality	Collection date; collector	Sample ID for DNA sequences	Nucleotide sequence accession number		
				Fungal 18S rRNA gene	Bacterial 16S rRNA gene	Insect 28S rRNA gene
Ledra auditura Walker	Oguni, Yamagata, Japan	Jul 2011; MT	ogn1-ogn6	LC108751-LC108756	–	LC108774-LC108779
	Oguni, Yamagata, Japan	Jul 2014; TF	ogn7-ogn8	LC108757-LC108758	–	LC108780-LC108781
Ledropsis discolor (Uhler)	Tsukuba, Ibaraki, Japan[a]	Nov 2014; MM	–	–	–	–
	Daigo, Ibaraki, Japan	May 2015; TN	dig1	LC108759	LC108768	LC108782
	Mt. Tsukuba, Ibaraki, Japan	May 2015; TF	tkb1	LC108760	LC108769	LC108783
	Mt. Takao, Tokyo, Japan	Jun 2015; TN	tko1-tko4	LC108761-LC108764	LC108770-LC108773	LC108784-LC108787
Tituria angulata (Matsumura)	Okinawa Isl., Okinawa, Japan	May 2011; TF	okn1	LC108765	–	LC108788
	Ishigaki Isl., Okinawa, Japan[a]	Jun 2012; TH	–	–	–	–
	Fushan, Yilan, Taiwan	Jul 2015; TF	fsn1	LC108766	–	LC108789
	Urao, Hsinchu, Taiwan	Jul 2015; TF	uro1	LC108767	–	LC108790

MM Minoru Moriyama, *MT* Masahiko Tanahashi, *TF* Takema Fukatsu, *TH* Takahiro Hosokawa, *TN* Takanori Nishino

[a] Used for histological examination only

TGA TCA TGG CTC AGG ATG-3′) and CFB1515R (5′-GTA CGG CTA CCT TGT TAC GAC TTA G-3′) (Moran et al. 2005) under a temperature profile of 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 5 min. A 1.5-kb region of the general bacterial 16S rRNA gene was amplified by PCR with primers 10F (5′-AGT TTG ATC ATG GCT CAG ATT G-3′) and 1507R (5′-TAC CTT GTT ACG ACT TCA CCC CAG-3′) (Sandström et al. 2001) under a temperature profile of 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 5 min. A 0.6-kb region of the insect 28S rRNA gene was amplified by PCR with primers Lalt (5′-CCT CGG ACC TTG AAA ATC C-3′) and Galt (5′-TGT CTC CTT ACA GTG CCA GA-3′) (Dietrich et al. 2001) under a temperature profile of 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 60 s, and a final extension at 72 °C for 5 min. After checking successful amplification by 1 % agarose gel electrophoresis, each PCR product was purified using exonuclease I (New England Biolabs) and shrimp alkaline phosphatase (Takara Bio) at 37 °C for 15 min followed by 80 °C for 15 min. The purified PCR products were directly subjected to a sequencing reaction using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed by an ABI PRISM 373 DNA Sequencer (Applied Biosystems). The following internal primers were used for sequencing: NS2 (5′-GGC TGC TGG CAC CAG ACT TGC-3′), NS3 (5′-GCA AGT CTG GTG CCA GCA GCC-3′), NS5 (5′-AAC TTA AAG GAA TTG ACG GAA G-3′) and NS7 (5′-GAG GCA ATA ACA GGT CTG TGA TGC-3′) for the fungal 18S rRNA gene (White et al. 1990) and 16SA2 (5′-GTG CCA GCA GCC GCG GTA ATA C-3′) and 16SA3 (5′-TGC ATG GYT GTC GTC AGC TCG-3′) for the bacterial 16S rRNA gene (Fukatsu and Nikoh 1998). From DNA samples of *L. discolor*, the fungal 18S rRNA gene fragment was not amplified by PCR despite the presence of the fungal symbiont, presumably because of inserted group I introns, which are frequently found in the ribosomal RNA genes of *Ophiocordyceps* fungi (Nikoh and Fukatsu 2001). Hence, the DNA samples, which also contained much ribosomal RNA in the absence of ribonuclease treatment, were reverse-transcribed with random nucleotide hexamers and SuperScript III Reverse transcriptase (Thermo Fisher Scientific), and the resultant complementary DNA samples were subjected to PCR amplification of fungal 18S rRNA gene.

Molecular phylogenetic analysis

The nucleotide sequences were multiple-aligned together with related nucleotide sequences retrieved from the DNA databases using the program ClustalW (Thompson et al. 1994). The raw alignments were edited manually to remove ambiguously aligned sites and gap-containing sites. Maximum-likelihood phylogenies were constructed using the program MEGA 6 (Tamura et al. 2013) under the Tamura-Nei substitution model and the Nearest-Neighbor-Interchange heuristic model with 1000 bootstrap replicates. Bayesian phylogenies were built by the program MrBayes (Huelsenbeck and Ronquist 2001). The best substitution models were selected using the program Kakusan 4 (Tanabe 2011) as K80 Gamma for the fungal 18S rRNA gene, GTR Gamma for the bacterial 16S rRNA gene and K80 Gamma for the insect 28S rRNA gene, respectively. Two replicate Bayesian analyses were performed, where four Markov chains were used in each replicate and the chain was sampled for every 100 interactions. The analyses were allowed to run for 1,000,000 generations, from which the first 100,000 generations of each replicate were excluded as burn in.

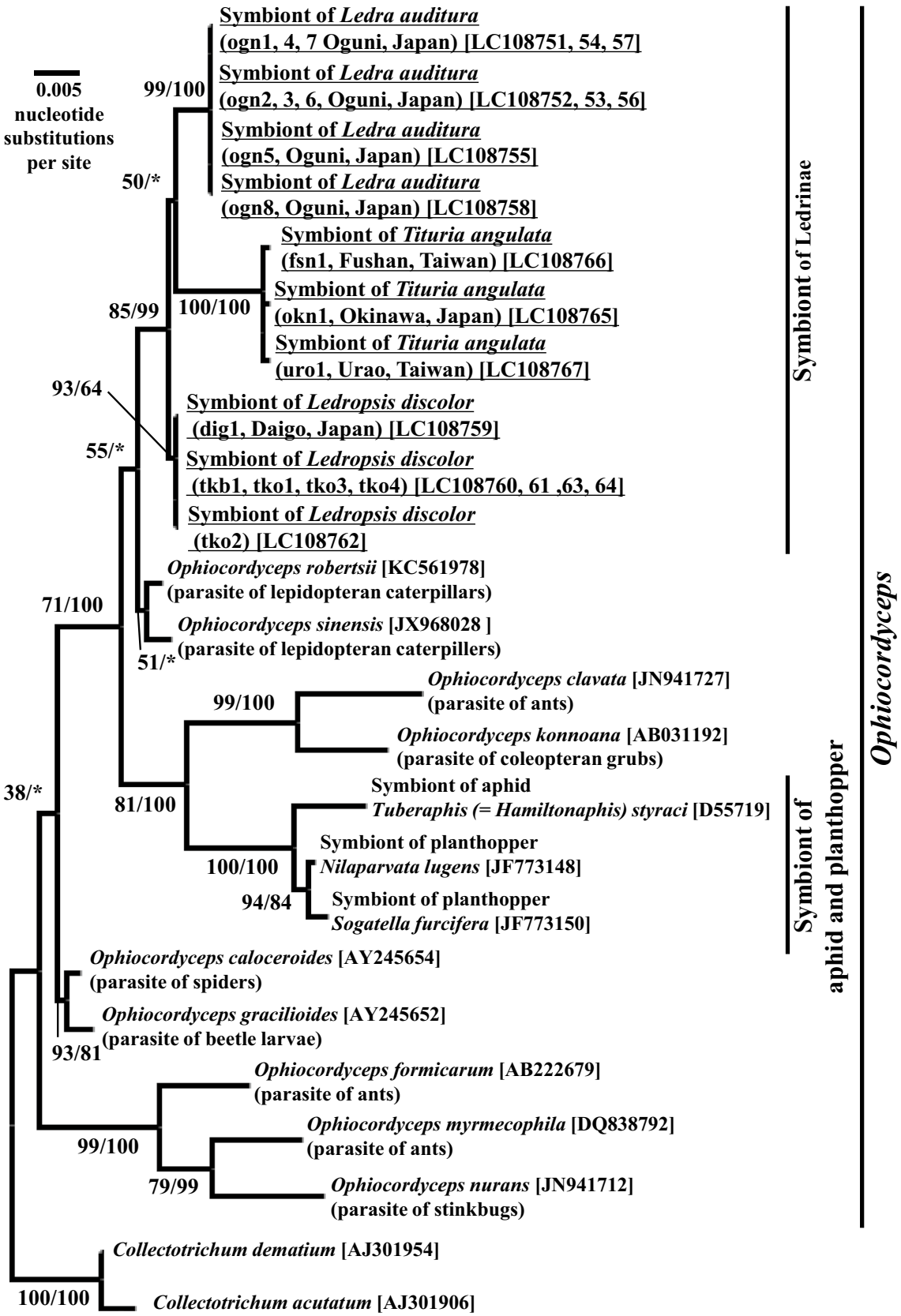
Fig. 2 Phylogenetic placement of the fungal symbionts associated with the eared leafhoppers *L. discolor*, *L. auditura* and *T. angulata* on the basis of fungal 18S rRNA gene sequences. A maximum-likelihood phylogeny inferred from 1389 aligned nucleotide sites is shown. Bootstrap probabilities of maximum-likelihood analysis and posterior probabilities of Bayesian analysis are indicated at the nodes, where *asterisks* imply that the nodes were not supported by the Bayesian model. A *scale bar* shows the branch length in terms of the number of substitutions per nucleotide site

Histology

For observation of the fungal symbiont cells, abdominal fat bodies were dissected from the acetone-preserved insects, crushed and suspended in a phosphate-buffered saline, mounted on a glass slide with a coverslip, and observed under a phase-contrast microscope. For localizing the fungal symbiont cells in the host tissues, the acetone-preserved insects were dissected in 70 % ethanol, and the dissected abdomens were fixed in ethanol-formalin (3:1). The fixed abdomens were cleared through an ethanol-xylene series, embedded in paraffin, processed into tissue sections (10 μm thick) and mounted on glass slides. The tissue sections were dewaxed and hydrated through a xylene-ethanol–water series, stained with periodic acid-Schiff (PAS) reagent, counter-stained with hematoxylin and observed under a light microscope.

In situ hybridization

Localization of bacterial symbionts in *L. discolor* was visualized by whole-mount in situ hybridization as previously described (Koga et al. 2009) using the following oligonucleotide probes targeting bacterial 16S rRNA: Sul664R (5′-Alexa647-CCM CAC ATT CCA GYT ACT CC-3′),


0.005
nucleotide
substitutions
per site
Symbiont of Ledra auditura
(ogn1, 4, 7 Oguni, Japan) [LC108751, 54, 57]
Symbiont of Ledra auditura
(ogn2, 3, 6, Oguni, Japan) [LC108752, 53, 56]
Symbiont of Ledra auditura
(ogn5, Oguni, Japan) [LC108755]
Symbiont of Ledra auditura
(ogn8, Oguni, Japan) [LC108758]
Symbiont of Tituria angulata
(fsn1, Fushan, Taiwan) [LC108766]
Symbiont of Tituria angulata
(okn1, Okinawa, Japan) [LC108765]
Symbiont of Tituria angulata
(uro1, Urao, Taiwan) [LC108767]
Symbiont of Ledropsis discolor
(dig1, Daigo, Japan) [LC108759]
Symbiont of Ledropsis discolor
(tkb1, tko1, tko3, tko4) [LC108760, 61 ,63, 64]
Symbiont of Ledropsis discolor
(tko2) [LC108762]
Ophiocordyceps robertsii [KC561978]
(parasite of lepidopteran caterpillars)
Ophiocordyceps sinensis [JX968028]
(parasite of lepidopteran caterpillers)
Ophiocordyceps clavata [JN941727]
(parasite of ants)
Ophiocordyceps konnoana [AB031192]
(parasite of coleopteran grubs)
Symbiont of aphid
Tuberaphis (= Hamiltonaphis) styraci [D55719]
Symbiont of planthopper
Nilaparvata lugens [JF773148]
Symbiont of planthopper
Sogatella furcifera [JF773150]
Ophiocordyceps caloceroides [AY245654]
(parasite of spiders)
Ophiocordyceps gracilioides [AY245652]
(parasite of beetle larvae)
Ophiocordyceps formicarum [AB222679]
(parasite of ants)
Ophiocordyceps myrmecophila [DQ838792]
(parasite of ants)
Ophiocordyceps nurans [JN941712]
(parasite of stinkbugs)
Collectotrichum dematium [AJ301954]
Collectotrichum acutatum [AJ301906]
99/100
50/*
85/99
100/100
93/64
55/*
71/100
51/*
99/100
38/*
81/100
100/100
94/84
93/81
99/100
79/99
100/100
Symbiont of Ledrinae
Symbiont of aphid and planthopper
Ophiocordyceps

which specifically detects *Sulcia* symbionts (Koga et al. 2013); BET940R (5′-Alexa488-TTA ATC CAC ATC ATC CAC CG-3′), which targets *Nasuia*-allied symbionts and other betaproteobacteria (Demanèche et al. 2008); EUB338 (5′-Alexa555-GCT GCC TCC CGT AGG AGT-3′), which universally recognizes eubacteria (Amann et al. 1990).

Nucleotide sequence accession numbers

The nucleotide sequences of the fungal 18S rRNA gene, bacterial 16S rRNA gene and insect 28S rRNA gene have been deposited in the DDBJ, EMBL and GenBank databases under accession numbers LC108751 to LC108790 (Table 1).

Results

Yeast-like fungal symbionts in the abdomen of eared leafhoppers

When the abdominal tissues of *L. discolor*, *L. auditura* and *T. angulata* were dissected, suspended and observed under a light microscope, numerous yeast-like particles were found in all the species and samples: slender particles in *L. discolor* (Fig. 1d); smaller round particles in *L. auditura* (Fig. 1e); larger round particles in *T. angulata* (Fig. 1f).

Fungal symbionts of eared leafhoppers are placed within the entomoparasitic fungal genus *Ophiocordyceps*

From all *L. auditura* and *T. angulata* samples, a 1.5-kb region of the fungal 18S rRNA gene was amplified by PCR. For all *L. discolor* samples, the PCR product was not obtained when the DNA samples were used as templates, but the 1.5-kb product was successfully amplified when reverse-transcribed complementary DNA samples were used as templates, presumably because the fungal 18S rRNA gene in the *L. discolor* samples contains group I introns (Nikoh and Fukatsu 2001). The 18S rRNA gene sequences were almost identical between individuals of the same species: from 99.9 % (1336/1338) to 100 % (1340/1340) between *L. discolor* samples and populations ($n = 6$); from 99.7 % (1338/1342) to 100 % (1340/1340) between *L. auditura* samples ($n = 8$); from 98.0 % (1312/1339) to 99.8 % (1336/1339) between Japanese and Taiwanese *T. angulata* populations ($n = 3$). Note that a *T. angulata* sample, uro1, contained a 25-bp gap, which is responsible for the exceptionally low sequence identity of 98.0 %. Molecular phylogenetic analysis revealed that the fungal symbionts of *L. discolor*, *L. auditura* and *T. angulata* formed a clade within the Ascomycota, where the clade of the ledrine fungal symbionts was placed within the entomoparasitic fungal genus *Ophiocordyceps* (Fig. 2).

L. discolor harbors the *Sulcia* symbiont, whereas *L. auditura* and *T. angulata* possess no bacterial symbiont

When the bacterial 16S rRNA gene was amplified by PCR with primers 10CFBFF and CFB1015R, which specifically target flavobacteria including *Sulcia* symbionts of diverse hemipteran insects (Moran et al. 2005), a PCR product of the expected size (1.5 kb) was obtained from all *L. discolor* samples, but not from *L. auditura* and *T. angulata* samples. The 16S rRNA gene sequences were identical between different samples and populations of *L. discolor*. Molecular phylogenetic analysis revealed that the *Sulcia* symbiont of *L. discolor* was phylogenetically allied to *Sulcia* symbionts of leafhoppers and treehoppers of the superfamily Membracoidea (Fig. 3). Whole-mount in situ hybridization with a *Sulcia*-specific probe Sul664R (Koga et al. 2013) identified localization of the *Sulcia* symbiont to a pair of bacteriomes in the abdomen (Fig. 4a), as observed in other leafhoppers (Bennett and Moran 2013; Ishii et al. 2013; Noda et al. 2012). On the other hand, when bacterial 16S rRNA gene was amplified by PCR with primers 10F and 1507R, which target diverse eubacteria in general but fail to detect *Sulcia* (Koga et al. 2013; Sandström et al. 2001), no PCR amplification was observed in any *L. discolor*, *L. auditura* and *T. angulata* samples. Whole-mount in situ hybridization with a general eubacteria probe EUB338 (Amann et al. 1990) detected a pair of abdominal bacteriomes in *L. discolor*, but no positive signals were observed in *L. auditura* and *T. angulata* (data not shown). Whole-mount in situ hybridization with a probe BET940R, which target *Nasuia*-allied symbionts of leafhoppers and other hemipterans (Demanèche et al. 2008; Koga et al. 2013), detected no symbiont signals in *L. discolor* (Fig. 4b), *L. auditula* and *T. angulata* (data not shown). Taken together, these results indicate that *L. discolor* harbors the *Sulcia* symbiont in the abdominal bacteriomes, while *L. auditura* and *T. angulata* possess no bacteriome-associated bacterial symbiont. It should be noted, however, that the possibility cannot be excluded that some minor bacterial associates may be present in some of the eared leafhoppers.

Localization of fungal symbiont and *Sulcia* symbiont in eared leafhoppers

Tissue sections of the eared leafhoppers were subjected to PAS staining, which histochemically visualizes polysaccharides in red. Since fungal cell walls usually contain large amounts of glucans, the fungal symbiont cells are highlighted in tissues and cells of the host insects by PAS staining (Noda 1977; Sasaki et al. 1996). In *L. discolor*, throughout the fat bodies

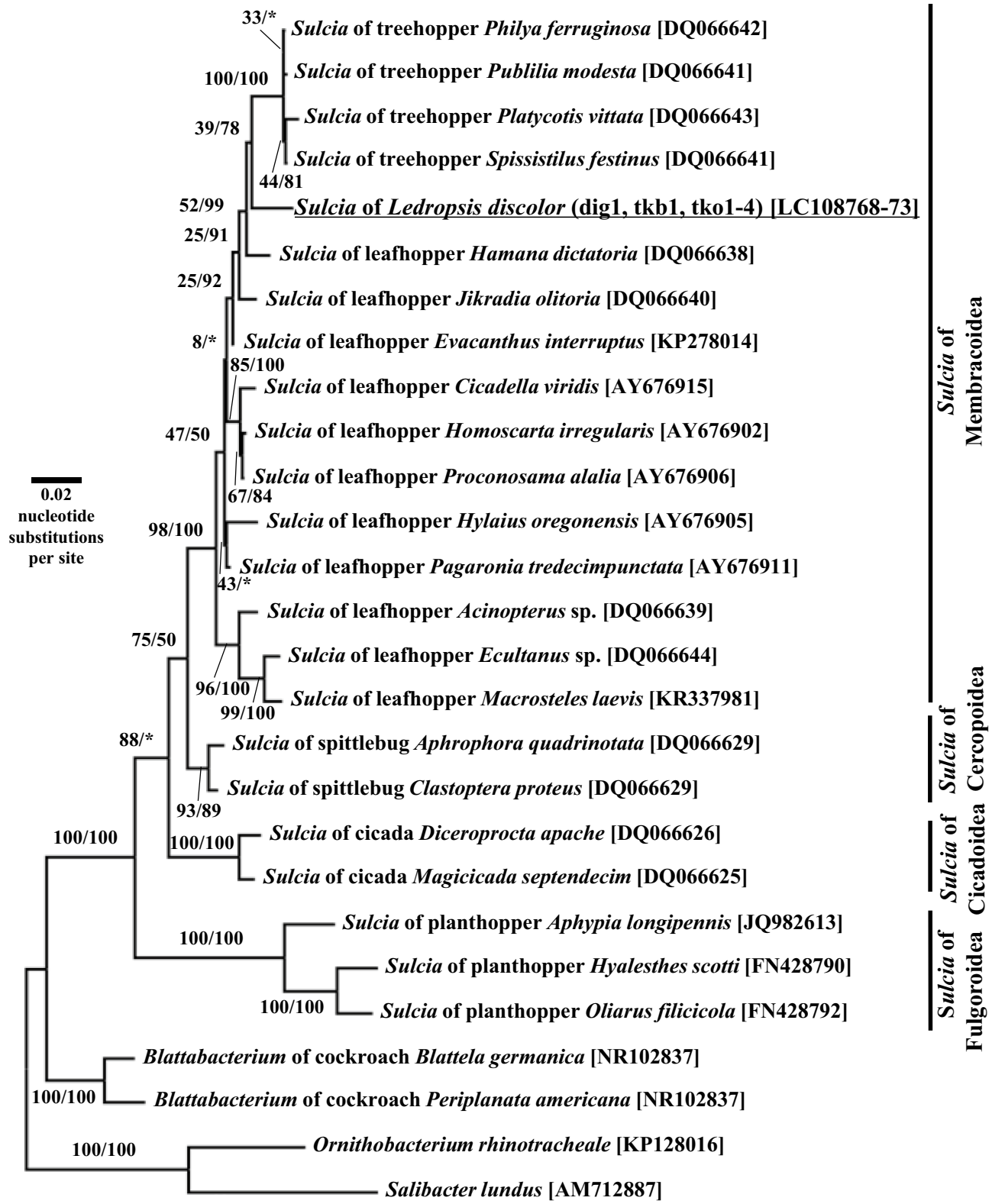


Fig. 3 Phylogenetic placement of the *Sulcia* symbiont associated with *L. discolor* on the basis of bacterial 16S rRNA gene sequences. A maximum-likelihood phylogeny inferred from 1111 aligned nucleotide sites is shown. Statistical supports and a *scale bar* are shown as in Fig. 2

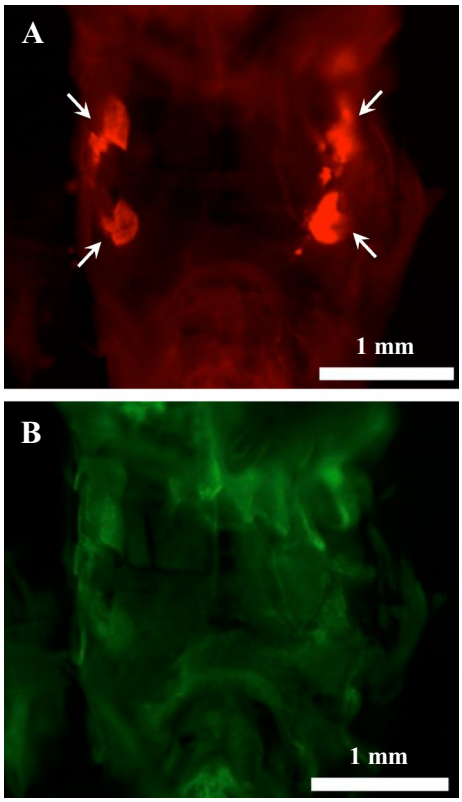


Fig. 4 Whole-mount in situ hybridization of bacterial symbionts in *L. discolor*. **a** With a *Sulcia*-specific probe Sul664R, hybridization signals in *red* are localized in a pair of bacteriomes in the abdomen. Note that each bacteriome is narrowed in the middle, consisting of anterior and posterior lobes (*arrows*). **b** With a betaproteobacterial probe BET940R, no hybridization signals were detected in the bacteriomes. Weak *green* signals are due to autofluorescence of insect tissues

distributed in the peripheral regions of the abdominal body cavity, some areas were represented by host cells that were densely populated by the fungal symbionts ("fhc" in Fig. 5a, b), whereas the *Sulcia*-harboring bacteriomes were located at a relatively central region of the abdominal body cavity ("bac" in Fig. 5a). In *L. auditura*, host cells populated by the fungal symbionts were scattered throughout the fat bodies located at the peripheral body cavity ("fhc" in Fig. 5c, d). In *T. angulata*, similarly, some host cells within the fat bodies were densely populated by the fungal symbionts ("fhc" in Fig. 5e). In addition, we observed that, in the nymphal specimen of *T. angulata*, host cells populated by the fungal symbionts formed clusters in relatively central regions of the abdominal body cavity close to the fat bodies ("fhc*" in Fig. 5e, f), although their cytological identity was obscure.

Phylogenetic relationship of eared leafhoppers

Figure 6 shows the phylogenetic relationship of the eared leafhoppers of the subfamily Ledrinae to other groups of leafhoppers and treehoppers on the basis of partial 28S rRNA gene sequences. The whole grouping of leafhoppers and treehoppers, namely the superfamily Membracoidea, was well supported. The eared leafhoppers formed a clade, which was allied to the leafhopper subfamily Cicadellinae. However, it should be noted that, considering the small sequence data and the low statistical supports for the groupings, the relationship between these insect groups is tentative, and further studies with more sequence data should be performed to obtain conclusive results (Cryan 2005; Cryan and Urban 2011).

Discussion

In this study, we examined the eared leafhoppers of the subfamily Ledrinae representing three genera, three species and nine local populations and consistently identified fungal symbionts from them (Figs. 1d–f, 2, 5), verifying an early histological report on the presence of a yeast-like fungal symbiont in a European ledrine species *L. aurita* (Buchner 1925). Molecular phylogenetic analysis revealed that the fungal symbionts form a clade within the ascomycetous genus *Ophiocordyceps* consisting of entomoparasitic fungi (Fig. 2). These results suggest the possibility that the fungal symbiont was acquired by the common ancestor of the subfamily Ledrinae and derived from an entomoparasite of the genus *Ophiocordyceps*.

The fungal genus *Ophiocordyceps* was recently divided from the genus *Cordyceps* (Sung et al. 2007). Conventionally, the entomoparasitic fungi of the genus *Cordyceps*, consisting of over 400 described species in the world and some 300 species in Japan, have been classified to the family Clavicipitaceae, the order Clavicipitales, the class Sordariomycetes and the phylum Ascomycota (Kobayashi 1982; Mains 1958; Shimizu 1994). On the basis of extensive molecular phylogenetic data, a recent revision of this fungal group re-organized the Hypocreales (formerly the Clavicipitales), whose members are mostly parasitic to arthropods, plants or fungi, into three major families, the Cordycipitaceae, Clavicipitaceae and Ophiocordycipitaceae (Sung et al. 2007). The family Ophiocordycipitaceae consists of two major genera: *Elaphocordyceps*, whose members are parasitic either to hart's truffles of the genus *Elaphomyces* or to cicada nymphs (Nikoh and Fukatsu 2000), and *Ophiocordyceps*, whose members are parasitic to diverse insects representing the orders Coleoptera (chafer grubs, click beetle larvae, ground beetles, etc.), Lepidoptera (moth larvae and pupae), Hymenoptera (ants and wasps) and Hemiptera (cicada nymphs, scale insects, spittlebugs, stinkbugs, etc.) (Sung et al. 2007). It seems plausible, although speculative, that a leafhopper-parasitizing *Ophiocordyceps* species stopped killing the host, persisted through host generations

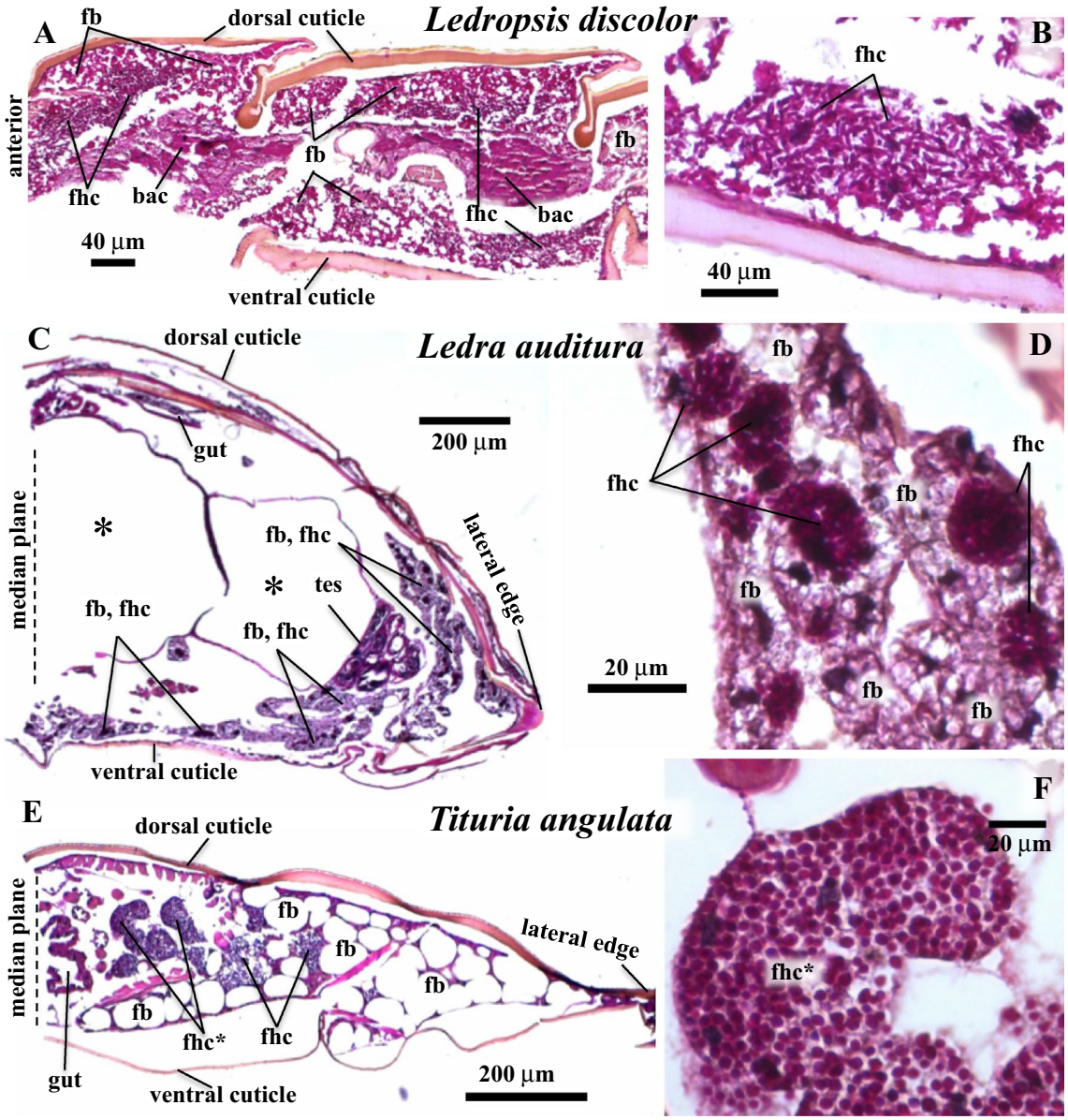


Fig. 5 Tissue sections of the eared leafhoppers *L. discolor*, *L. auditura* and *T. angulata* subjected to PAS staining. **a** An abdominal sagittal section of an *L. discolor* nymph in which a stretch of bacteriome and fungus-harboring cells in the fat body are seen. **b** An enlarged image of the fungus-harboring cells of the *L. discolor* nymph, whose cytoplasm is full of the fungal symbionts. **c** An abdominal cross section of a *L. auditura* adult. **d** An enlarged image of the fat body in the *L. auditura* adult, in which a number of fungus-harboring cells are observed. **e** An abdominal cross section of a *T. angulata* nymph, in which clusters of fungus-harboring cells are seen. **f** An enlarged image of the fungus-harboring cells in the abdominal body cavity of the *T. angulata* nymph, whose cytoplasm is filled with the fungal symbionts. *bac* bacteriome, *fb* fat body, *fhc* fungus-harboring cells the in fat body, *fhc** fungus-harboring cells in the central body cavity adjacent to the fat body, *tes* testicule. *Asterisks* in (**c**) indicate tissue/cell-free spaces in the body cavity, which are found in adult insects of the eared leafhoppers

via vertical transmission, exerted positive effects on the host fitness, became a mutualistic partner and took over the bacteriome-associated symbionts in the Ledrinae.

In this context, it is notable that such symbiont replacements from bacteria to fungi must have occurred repeatedly in the Hemiptera. For example, while the majority of over 4000 aphid species in the world are associated with the obligate bacterial symbiont *Buchnera* (Baumann 2005; Douglas 1998; Moran et al. 2008), a small number of aphids of the genera *Tuberaphis*, *Cerataphis* and *Glyphinaphis* in the tribe Cerataphidini lack *Buchnera* and harbor yeast-like fungal symbionts (Fukatsu and Ishikawa 1992, 1996; Fukatsu et al. 1994; Hongoh and Ishikawa 2000). Although some planthoppers of the superfamily

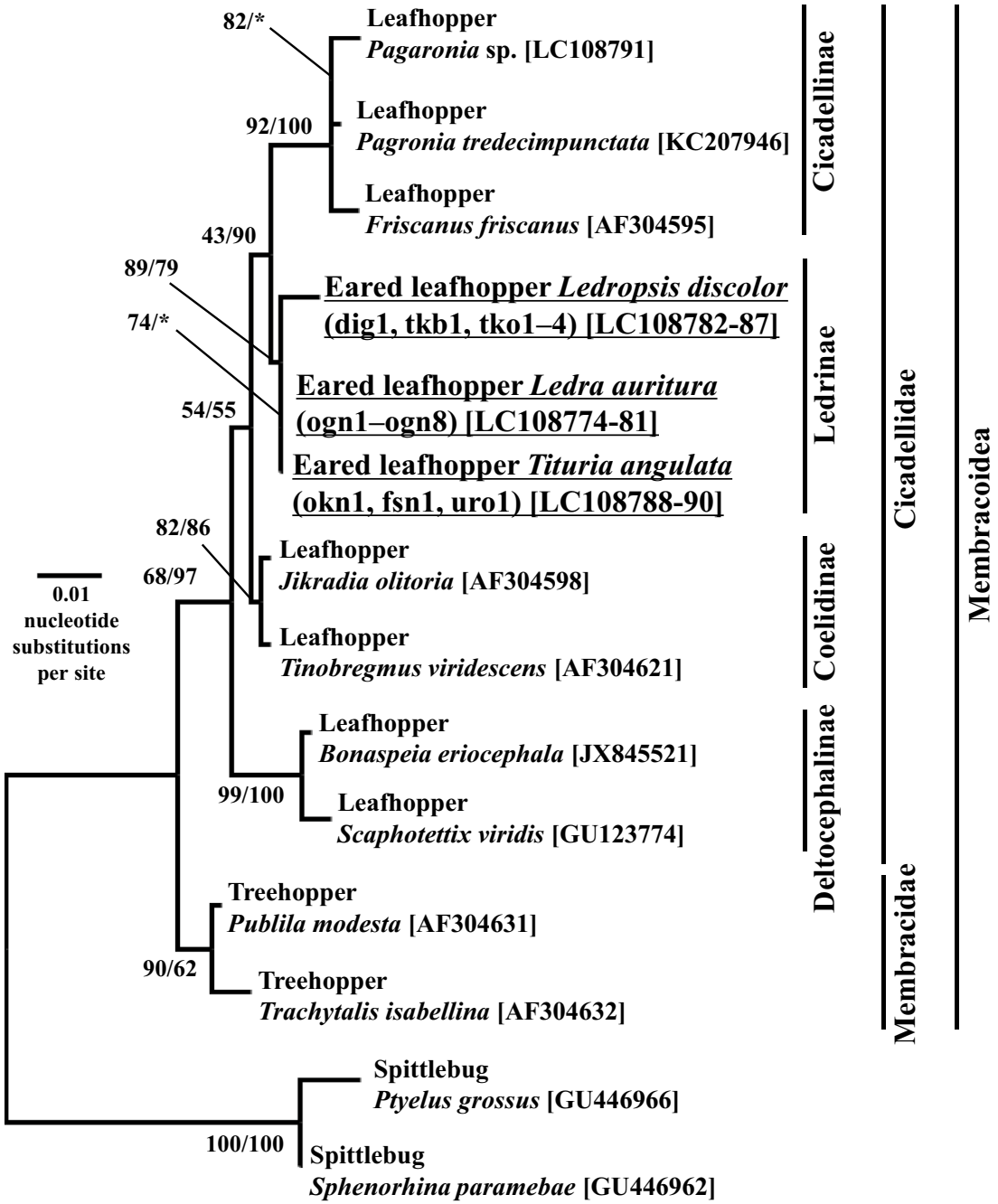


Fig. 6 Phylogenetic placement of the eared leafhoppers *L. discolor*, *L. auditura* and *T. angulata* among leafhoppers and treehoppers of the superfamily Membracoidea. A maximum-likelihood phylogeny inferred from 634 aligned nucleotide sites of insect 28S rRNA gene sequences is shown. Statistical supports and a *scale bar* are shown as in Fig. 2

Fulgoroidea are associated with *Sulcia*, *Vidania* and other bacterial symbionts (Bressan and Mulligan 2013; Bressan et al. 2009; Gonella et al. 2008, 2011; Urban and Cryan 2012), other planthopper species are devoid of the bacterial symbionts and associated with yeast-like fungal symbionts (Michalik et al. 2009; Noda 1977; Noda et al. 1995; Sasaki et al. 1996; Suh et al. 2001; Szklarzewicz et al. 2007). Even in the leafhopper family Cicadellidae, some species such as *Scaphoideus titanus* Ball possess neither *Sulcia* nor *Nasuia*, but harbor a yeast-like fungal symbiont (Sacchi et al. 2008). In the eared leafhoppers of the subfamily Ledrinae, we found that *L. discolor* is associated with both *Sulcia* and a fungal symbiont (Figs. 2, 3, 5), which provides an insight into the evolutionary process of the symbiont replacement. Here we hypothesize that, in the common ancestor of the Ledrinae, the newcomer fungal symbiont first replaced the

bacteriome-associated *Nasuia*-allied co-symbiont while retaining the ancient *Sulcia* symbiont, and subsequently also took over *Sulcia*, thereby completing the replacement from the prokaryotic symbiosis to the eukaryotic one. Similar stepwise processes of symbiont replacements might have also occurred in planthoppers (from *Sulcia* and *Vidania* to the fungal symbiont) and other hemipteran groups (Buchner 1965; Müller 1962).

The molecular phylogeny clearly showed that the fungal symbionts of the eared leafhoppers are not allied to the fungal symbionts of aphids and planthoppers (Fig. 2). This phylogenetic pattern favors the idea of multiple evolutionary origins of the fungal symbionts from the genus *Ophiocordyceps* within the Hemiptera, and highlights the evolutionary connection between parasitism and mutualism in the insect-microbe symbiotic associations. It should be noted that the fungal symbiont of aphids is phylogenetically very close to the fungal symbiont of leafhoppers, although their evolutionary origins must be independent (Hongoh and Ishikawa 2000) (see Fig. 2), suggesting the possibility that particular groups of *Ophiocordyceps* fungi specialized for hemipteran insects might recurrently serve as evolutionary sources of the fungal symbionts in the Hemiptera. In this context, the possibility of multiple independent fungal acquisitions from closely related entomoparasitic *Ophiocordyceps* fungi in the Ledrinae should also be kept in mind.

Biological functions of the fungal symbiont in the eared leafhoppers are currently unknown. Genomics of *Sulcia* and co-symbionts have suggested that these bacterial symbionts cooperatively provide essential amino acids and other nutrients for leafhoppers and other hemipteran insect hosts (Bennett and Moran 2015; McCutcheon and Moran 2007, 2012; McCutcheon et al. 2009; McCutcheon and Moran 2010; Wu et al. 2006). Hence, the fungal symbiont must play similar nutritional roles in place of the bacterial symbionts. Considering the much larger genome size and consequent broader metabolic capability of the fungal symbiont in comparison with the tiny-genome bacterial symbionts (McCutcheon 2010; Moran and Bennett 2014; Vogel and Moran 2013; Xue et al. 2015), it is conceivable, although speculative, that the fungal symbiont may play additional biological roles in the hemipteran hosts. Genomics of the fungal symbionts (Vogel and Moran 2013; Xue et al. 2015) and physiological studies on normal and fungus-deprived insect hosts (Noda and Saito 1979; Sasaki et al. 1996) are needed for deeper understanding of functional aspects of the insect-fungus symbiotic association.

The subfamily Ledrinae is a relatively small leafhopper group consisting of some 38 genera and 300 species (Jones and Deitz 2009). In this study, we inspected only three ledrine species representing three genera. Our preliminary phylogenetic analysis confirmed the monophyly of the ledrine leafhoppers but did not resolve their phylogenetic relationship (Fig. 6). Hence, we cannot discuss the detailed evolutionary processes as to when and how the *Ophiocordyceps* fungus invaded the symbiotic system, replaced the *Nasuia*-allied co-symbiont, and finally took over the ancient *Sulcia* symbiont. To address these evolutionary issues, broader taxon sampling of the ledrine leafhoppers and more DNA sequence data are needed. Considering the phylogenetic and structural integrity between the ledrine leafhoppers and their fungal symbionts (Figs. 2, 5), the fungal symbiont must have been stably maintained through host generations by vertical transmission via ovarial passage as observed in planthoppers and other hemipterans (Buchner 1925, 1965; Cheng and Hou 2001; Michalik et al. 2009; Noda 1977; Szklarzewicz et al. 2007; Yukuhiro et al. 2014). To observe the symbiont transmission process in the ledrine leafhoppers, we should inspect the insect samples with mature ovaries containing developing oocytes, which were unfortunately not obtained in this study.

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References

Amann RI, Krumholz L, Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770

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

Bennett GM, Moran NA (2013) Small, smaller, smallest: the origins and evolution of ancient dual symbioses in a phloem-feeding insect. Genome Biol Evol 5:1675–1688

Bennett GM, Moran NA (2015) Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. Proc Natl Acad Sci 112:10169–10176

Bennett GM, McCutcheon JP, MacDonald BR, Romanovicz D, Moran NA (2014) Differential genome evolution between companion symbionts in an insect-bacterial symbiosis. Mbio 5(5):e01697–e01714

Bennett GM, McCutcheon JP, Mcdonald BR, Moran NA (2016) Lineage-specific patterns of genome deterioration in obligate symbionts of sharpshooter leafhoppers. Genome Biol Evol 8(1):296–301

Bourtzis K, Miller TA (2003) Insect symbiosis. CRC Press, Boca Raton

Bressan A, Mulligan KL (2013) Localization and morphological variation of three bacteriome-inhabiting symbionts within a planthopper of the genus *Oliarus* (Hemiptera: Cixiidae). Environ Microbiol Rep 5:499–505

Bressan A, Arneodo J, Simonato M, Haines WP, Boudon-Padieu E (2009) Characterization and evolution of two bacteriome-inhabiting symbionts in cixiid planthoppers (Hemiptera: Fulgoromorpha: Pentastirini). Environ Microbiol 11:3265–3279

Buchner P (1925) Studien an intracellularen Symbionten V. Die symbiontischen Einrichtungen der Zikaden. Z Morphol Ökol Tiere 4:88–245

Buchner P (1965) Endosymbiosis of animals with plant microorganisms. Interscience, New York

Cheng DJ, Hou RF (2001) Histological observations on transovarial transmission of a yeast-like symbiote in *Nilaparvata lugens* Stal (Homoptera, Delphacidae). Tissue Cell 33:273–279

Cryan JR (2005) Molecular phylogeny of Cicadomorpha (Insecta: Hemiptera: Cicadoidea, Cercopoidea and Membracoidea): adding evidence to the controversy. Syst Entomol 30:563–574

Cryan JR, Urban JM (2011) Higher-level phylogeny of the insect order Hemiptera: is Auchenorrhyncha really paraphyletic? Syst Entomol 37:7–21

Demanèche S, Sanguin H, Poté J, Navarro E, Bernillon D, Mavingui P, Wildi W, Vogel TM, Simonet P (2008) Antibiotic-resistant soil bacteria in transgenic plant fields. Proc Natl Acad Sci 105:3957–3962

Dietrich CH, Rakitov RA, Holmes JL, Black WC 4th (2001) Phylogeny of the major lineages of Membracoidea (Insecta: Hemiptera: Cicadomorpha) based on 28S rDNA sequences. Mol Phylogenet Evol 18:293–305

Douglas AE (1988) Experimental studies on the mycetome symbiosis in the leafhopper *Euscelis incisus*. J Insect Physiol 34:1043–1053

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

Forero D (2008) The systematics of the Hemiptera. Rev Colomb Entomol 34:1–21

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

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

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

Gonella E, Negri I, Marzorati M, Brusetti L, Pajoro M, Mandrioli M, Tedeschi R, Daffonchio D, Alma A (2008) Study of the bacterial community affiliated to *Hyalesthes obsoletus*, the insect vector of "bois noir" phytoplasma of grape. Bull Insectol 61:221–222

Gonella E, Negri I, Marzorati M, Mandrioli M, Sacchi L, Pajoro M, Crotti E, Rizzi A, Clementi E, Tedeschi R, Bandi C, Alma A, Daffonchio D (2011) Bacterial endosymbiont localization in *Hyalesthes obsoletus*, the insect vector of Bois noir in *Vitis vinifera*. Appl Environ Microbiol 77:1423–1435

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

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755

Ishii Y, Matsuura Y, Kakizawa S, Nikoh N, Fukatsu T (2013) Diversity of bacterial endosymbionts associated with *Macrosteles* leafhoppers vectoring phytopathogenic phytoplasmas. Appl Environ Microbiol 79:5013–5022

Jones JR, Deitz LL (2009) Phylogeny and systematics of the leafhopper subfamily Ledrinae (Hemiptera: Cicadellidae). Zootaxa 2186:1–120

Kikuchi Y (2009) Endosymbiotic bacteria in insects: their diversity and culturability. Microb Environ 24:195–204

Kobayashi Y (1982) Keys to the taxa of the genera *Cordyceps* and *Torrubiella*. Trans Mycol Soc Jpn 23:329–364

Kobialka M, Michalik A, Walczak M, Junkiert Ł, Szklarzewicz T (2016) *Sulcia* symbiont of the leafhopper *Macrosteles laevis* (Ribaut, 1927) (Insecta, Hemiptera, Cicadellidae: Deltocephalinae) harbors *Arsenophonus* bacteria. Protoplasma 253:903–912

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

Koga R, Bennett GM, Cryan JR, Moran NA (2013) Evolutionary replacement of obligate symbionts in an ancient and diverse insect lineage. Environ Microbiol 15:2073–2081

Mains EB (1958) North American entomogenous species of *Cordyceps*. Mycologia 50:169–222

McCutcheon JP (2010) The bacterial essence of tiny symbiont genomes. Curr Opin Microbiol 13:73–78

McCutcheon JP, Moran NA (2007) Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci 104:19392–19397

McCutcheon JP, Moran NA (2010) Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. Genome Biol Evol 2:708–718

McCutcheon JP, Moran NA (2012) Extreme genome reduction in symbiotic bacteria. Nat Rev Microbiol 10:13–26

McCutcheon JP, McDonald BR, Moran NA (2009) Convergent evolution of metabolic roles in bacterial co-symbionts of insects. Proc Natl Acad Sci 106:15394–15399

Michalik A, Jankowska W, Szklarzewicz T (2009) Ultrastructure and transovarial transmission of endosymbiotic microorganisms in *Conomelus anceps* and *Metcalfa pruinosa* (Insecta, Hemiptera, Fulgoromorpha). Folia Biol (Krakow) 57:131–137

Michalik A, Jankowska W, Kot M, Gołas A, Szklarzewicz T (2014) Symbiosis in the green leafhopper, *Cicadella viridis* (Hemiptera, Cicadellidae). Association in statu nascendi? Arthropod Struct Dev 43:579–587

Moran NA, Bennett GM (2014) The tiniest tiny genomes. Annu Rev Microbiol 68:195–215

Moran NA, Dale C, Dunbar H, Smith WA, Ochman H (2003) Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome. Environ Microbiol 5:116–126

Moran NA, Tran P, Gerardo NM (2005) Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. Appl Environ Microbiol 71:8802–8810

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

Müller HJ (1962) Neuere Vorstellungen über Verbreitung und Phylogenie der Endosymbiosen der Zikaden. Z Morphol Ökol Tiere 51:190–210

Nikoh N, Fukatsu T (2000) Interkingdom host jumping underground: phylogenetic analysis of entomoparasitic fungi of the genus *Cordyceps*. Mol Biol Evol 17:629–638

Nikoh N, Fukatsu T (2001) Evolutionary dynamics of multiple group I introns in nuclear ribosomal RNA genes of endoparasitic fungi of the genus *Cordyceps*. Mol Biol Evol 18:1631–1642

Noda H (1977) Histological and histochemical observation of intracellular yeastlike symbiotes in the fat body of the smaller brown planthopper, *Laodelphax striatellus* (Homoptera: Delphacidae). Appl Entomol Zool 12:134–141

Noda H, Saito T (1979) The role of intracellular yeastlike symbiotes in the development of *Laodelphax striatellus* (Homoptera: Delphacidae). Appl Entomol Zool 14:453–458

Noda H, Nakashima N, Koizumi M (1995) Phylogenetic position of yeast-like symbiotes of rice planthoppers based on partial 18S rDNA sequences. Insect Biochem Mol Biol 25:639–646

Noda H, Watanabe K, Kawai S, Yukuhiro F, Miyoshi T, Tomizawa M, Koizumi Y, Nikoh N, Fukatsu T (2012) Bacteriome-associated endosymbionts of the green rice leafhopper *Nephotettix cincticeps* (Hemiptera: Cicadellidae). Appl Entomol Zool 47:217–225

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

Sacchi L, Genchi M, Clementi E, Bigliardi E, Avanzati AM, Pajoro M, Negri I, Marzorati M, Gonella E, Alma A, Daffonchio D, Bandi C (2008) Multiple symbiosis in the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae): details of transovarial transmission of *Cardinium* sp. and yeast-like endosymbionts. Tissue Cell 40:231–242

Salem H, Florez L, Gerardo N, Kaltenpoth M (2015) An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. Proc Biol Sci 282:201442957

Sandström JP, Russell JA, White JP, Moran NA (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. Mol Ecol 10:217–228

Sasaki T, Kawamura M, Ishikawa H (1996) Nitrogen recycling in the brown planthopper, *Nilaparvata lugens*: involvement of yeast-like endosymbionts in uric acid metabolism. J Insect Physiol 42:125–129

Shimizu D (1994) Color iconography of vegetable wasps and plant worms. Seibundo Shinkosha, Tokyo

Suh SO, Noda H, Blackwell M (2001) Insect symbiosis: derivation of yeast-like endosymbionts within an entomopathogenic filamentous lineage. Mol Biol Evol 18:995–1000

Sung GH, Hywel-Jones NL, Sung JM, Luangsa-Ard JJ, Shrestha B, Spatafora JW (2007) Phylogenetic classification of *Cordyceps* and the Clavicipitaceous fungi. Stud Mycol 57:5–59

Szklarzewicz T, Jankowska W, Lukasiewicz K, Szymańska B (2007) Structure of the ovaries and oogenesis in *Cixius nervosus* (Cixiidae), *Javesella pellucida* and *Conomelus anceps* (Delphacidae) (Insecta, Hemiptera, Fulgoromorpha). Arthropod Struct Dev 36:199–207

Takiya DM, Tran PL, Dietrich CH, Moran NA (2006) Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. Mol Ecol 15:4175–4191

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

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 Res 11:914–921

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 22:4673–4680

Urban JM, Cryan JR (2012) Two ancient bacterial endosymbionts have coevolved with the planthoppers (Insecta: Hemiptera: Fulgoroidea). BMC Evol Biol 12:87

Vogel KJ, Moran NA (2013) Functional and evolutionary analysis of the genome of an obligate fungal symbiont. Genome Biol Evol 5:891–904

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–751

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322

Wu D, Daugherty SC, Aken SEV, Pai GH, Watkins KL, Khouri H, Tallon LJ, Zaborsky JM, Dunbar HE, Tran PL, Moran NA, Eisen JA (2006) Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. PLoS Biol 4:e188

Xue J et al (2015) Genomes of the rice pest brown planthopper and its endosymbionts reveal complex complementary contributions for host adaptation. Genome Biol 15:521

Yukuhiro F, Miyoshi T, Noda H (2014) Actin-mediated transovarial transmission of a yeastlike symbiont in the brown planthopper. J Insect Physiol 60:111–117