Recurrent symbiont recruitment from fungal parasites in cicadas

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Diverse insects are associated with ancient bacterial symbionts, whose genomes have often suffered drastic reduction and degeneration. In extreme cases, such symbiont genomes seem almost unable to sustain the basic cellular functioning, which comprises an open question in the evolution of symbiosis. Here, we report an insect group wherein an ancient symbiont lineage suffering massive genome erosion has experienced recurrent extinction and replacement by host-associated pathogenic microbes. Cicadas are associated with the ancient bacterial co-obligate symbionts *Sulcia* and *Hodgkinia*, whose streamlined genomes are specialized for synthesizing essential amino acids, thereby enabling the host to live on plant sap. However, our inspection of 24 Japanese cicada species revealed that while all species possessed *Sulcia*, only nine species retained *Hodgkinia*, and their genomes exhibited substantial structural instability. The remaining 15 species lacked *Hodgkinia* and instead harbored yeast-like fungal symbionts. Detailed phylogenetic analyses uncovered repeated *Hodgkinia*-fungus and fungus-fungus replacements in cicadas. The fungal symbionts were phylogenetically intermingled with cicada-parasitizing *Ophiocordyceps* fungi, identifying entomopathogenic origins of the fungal symbionts. Most fungal symbionts of cicadas were uncultivable, but the fungal symbiont of *Meimuna opalifera* was cultivable, possibly because it is at an early stage of fungal symbiont replacement. Genome sequencing of the fungal symbiont revealed its metabolic versatility, presumably capable of synthesizing almost all amino acids, vitamins, and other metabolites, which is more than sufficient to compensate for the *Hodgkinia* loss. These findings highlight a straightforward ecological and evolutionary connection between parasitism and symbiosis, which may provide an evolutionary trajectory to renovate deteriorated ancient symbiosis via pathogen domestication.

cicadas | *Ophiocordyceps* | parasitic fungi | symbiotic fungi | symbiont replacement

Diverse insects are symbiotically associated with diverse microbes (1–3). In particular, extremely intimate relationships are found among host-symbiont associations underpinning stringent ecological and physiological necessities for energy, metabolites, or nutrients. For example, the majority of plant-sucking insects of the order Hemiptera, including aphids, whiteflies, scale insects, psyllids, cicadas, spittlebugs, leafhoppers, planthoppers, and many others, are obligatorily dependent on symbiotic microorganisms for provisioning of essential amino acids and other nutrients that are deficient in their sole food source of plant sap (4–7). In most cases, the hosts have developed specialized cells and organs, called bacteriocytes and bacteriomes, to which their specific symbionts are localized (8, 9). In the maternal body, the symbionts migrate to developing oocytes, thereby ensuring vertical symbiont transmission through host generations (10, 11). In many cases, the symbiont phylogeny mirrors the host phylogeny, indicating strict host-symbiont cospeciation over evolutionary time, which may exceed 100–200 million y (12, 13).

Notably, such intimate host-symbiont associations certainly entail stability and continuity on one hand, but, on the other hand, theoretical and empirical studies have shown that such host-symbiont associations may potentially suffer instability and collapse in the long run (14, 15). In obligate and long-lasting symbiotic associations, the symbiont genomes tend to exhibit drastic size reductions and massive gene losses, which are driven by relaxed natural selection acting on many symbiont genes unnecessary for the symbiotic lifestyle, and also by accumulation of deleterious mutations due to genetic drift facilitated by strong population bottlenecks and a paucity of horizontal gene acquisitions inherent in the obligate intrahost lifestyle (16–18). Some insect symbiont genomes are extremely reduced to 0.2 Mb or smaller in size, encode less than 200 genes, and so have genomes even smaller than some organellar genomes (19–22). By accumulating numerous mutations that could potentially lead to genomic malfunctioning and instability, such tiny-genome symbionts,

Significance

Cicadas are dependent on the essential bacterial symbionts *Sulcia* and *Hodgkinia*. The symbiont genomes are extremely streamlined for provisioning of essential amino acids and other nutrients. In some cicada lineages, *Hodgkinia* genomes are fragmented into numerous minicircles, which may represent a critical stage of genomic erosion close to collapse. What would happen subsequently? Our survey of the Japanese cicada diversity revealed that while *Sulcia* is conserved among all species, the majority of them have lost *Hodgkinia* and instead harbor yeast-like fungal associates. The fungal symbionts are phylogenetically intermingled with cicada-parasitizing *Ophiocordyceps* fungi, indicating recurrent symbiont replacements by entomopathogens in cicadas and providing insights into the mechanisms underlying the parasitism-symbiosis evolutionary continuum, compensation of symbiont genome erosion, and diversification of host-symbiont associations.

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Data deposition: The sequences reported in this paper have been deposited in the DNA Data Bank Japan Read Archive, www.ddbj.nig.ac.jp (accession nos. LC370451–LC371030) and the GenBank database www.ncbi.nlm.nih.gov/genbank/ (accession nos. MG737715–MG737734, CP029009–CP029028, SAMN08930808–SAMN08930810, and SAMN08939728–SAMN08939730; BioProject nos. PRJNA450103, PRJNA450106, PRJNA450107, PRJNA450109–PRJNA450112, PRJNA450114–PRJNA450119, PRJNA450122–PRJNA450127, PRJNA450129, and PRJNA427071).

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and potentially their hosts, may be near the edge of extinction due to genome erosion (14, 15). There are many examples, however, as in aphids (23–28), scale insects (29, 30), spittlebugs (31, 32), leafhoppers (33–37), planthoppers (38, 39), weevils (40, 41), lice (42, 43), and others (1, 44, 45), wherein an ancient and presumably degraded bacterial symbiont with essential biological function has been lost and replaced by totally different microbial associates. Whether the degenerative trend of symbiont genome evolution is relevant to the symbiont losses, replacements, and diversification, and if so, how, is mostly unanswered but remains an intriguing issue of evolutionary biology.

In this context, a relevant case of such symbiont genome degeneration may be observed in the bacterial cosymbionts of singing cicadas, *Sulcia* and *Hodgkinia*. *Sulcia* has a small genome of less than 0.3 Mb in size and comprises an ancient and conserved symbiont lineage, whose evolutionary origin dates back to the common ancestor of the Auchenorrhyncha (cicadas, spittlebugs, leafhoppers, planthoppers, etc.) as long as 260 million y ago (13, 46). By contrast, *Hodgkinia* is restricted to cicadas, indicating a relatively younger evolutionary origin than *Sulcia*, but its genome is even more drastically reduced, typically smaller than 0.15 Mb (47, 48). The *Sulcia* genome encodes biosynthetic pathway genes for most essential amino acids, while the *Hodgkinia* genome complementarily retains genes for the essential amino acids histidine and methionine and the vitamins cobalamin and riboflavin, thereby jointly supporting the growth and survival of host cicadas feeding solely on nutritionally deficient plant xylem sap (48, 49). Notably, in some cicada lineages, *Hodgkinia* has evolved into complexes of distinct cellular lineages with even more reduced but complementary genomes, which is interpreted as an unusual means of further genomic degradation (50–53). In extreme cases, the symbiont genome is broken down into an assemblage of dozens of minicircles, each encoding only a few genes, which may be leading to some critical stage of genomic instability (51–53). What, then, might be the fate of the *Sulcia-Hodgkinia*-cicada cosymbiotic association if indeed the genome complexity observed in *Hodgkinia* is nonadaptive or even maladaptive for the symbiosis?

Here, we report that frequent losses of *Hodgkinia* have certainly occurred in the natural cicada diversity. Our survey of 24 Japanese cicada species revealed that the majority, 15 species, lack *Hodgkinia* infection. *Hodgkinia* losses are estimated to have occurred repeatedly, at least three times and likely more. Strikingly, all of the *Hodgkinia*-free cicada species are associated with yeast-like fungal symbionts, uncovering recurrent evolutionary transitions from *Sulcia-Hodgkinia*-cicada symbiosis to *Sulcia*-fungus-cicada symbiosis. Phylogenetically, the fungal symbionts of cicadas are intermingled with cicada-parasitizing *Ophiocordyceps* fungi, identifying the evolutionary source of the fungal symbionts as the fungal parasites of cicadas. These results highlight a straightforward evolutionary connection between parasitism and symbiosis, and unveil an evolutionary trajectory to compensate for a deteriorating ancient bacterial symbiont by domesticated entomopathogens.

Results and Discussion

General Features of Symbiotic Organs in Japanese Cicadas. The superfamily Cicadoidea (Hemiptera: Auchenorrhyncha) includes over 3,000 species of large-sized, plant-sucking insects known as singing cicadas, and consists of two families (Cicadidae and Tettigarctidae) and several subfamilies (54). From the Japanese Archipelago, 1 family (Cicadidae), 2 subfamilies (Cicadinae and Cicadettinae), 15 genera, and 35 species of cicadas have been described (55), of which we collected adult insects of 24 species representing 13 genera (*SI Appendix*, Table S1). In the abdominal body cavity, in addition to gonads, fat bodies, and an alimentary tract, voluminous tissue masses resembling grape bunches, colored white, pink or yellow, were consistently observed, which represented the symbiotic organs, called the bacteriomes, of the cicadas (Fig. 1).

Endosymbiotic Microbiota in Japanese Cicadas. The bacteriomes and other tissues were dissected from the cicada samples and subjected to PCR amplification/cloning/sequencing/detection of the bacterial 16S rRNA gene for all 24 species representing 73 populations and 219 individuals (*SI Appendix*, Table S1). Among them, dissected bacteriomes, often associated with fat body fragments, from 20 samples representing 20 species were subjected to metagenomic Illumina sequencing. In the metagenomic assemblies, we identified mostly complete coding regions of mitochondrial genomes of host cicadas, complete *Sulcia* genome sequences, and genomic contigs of *Hodgkinia* and other microbial associates (*SI Appendix*, Fig. S1 and Tables S2–S5).

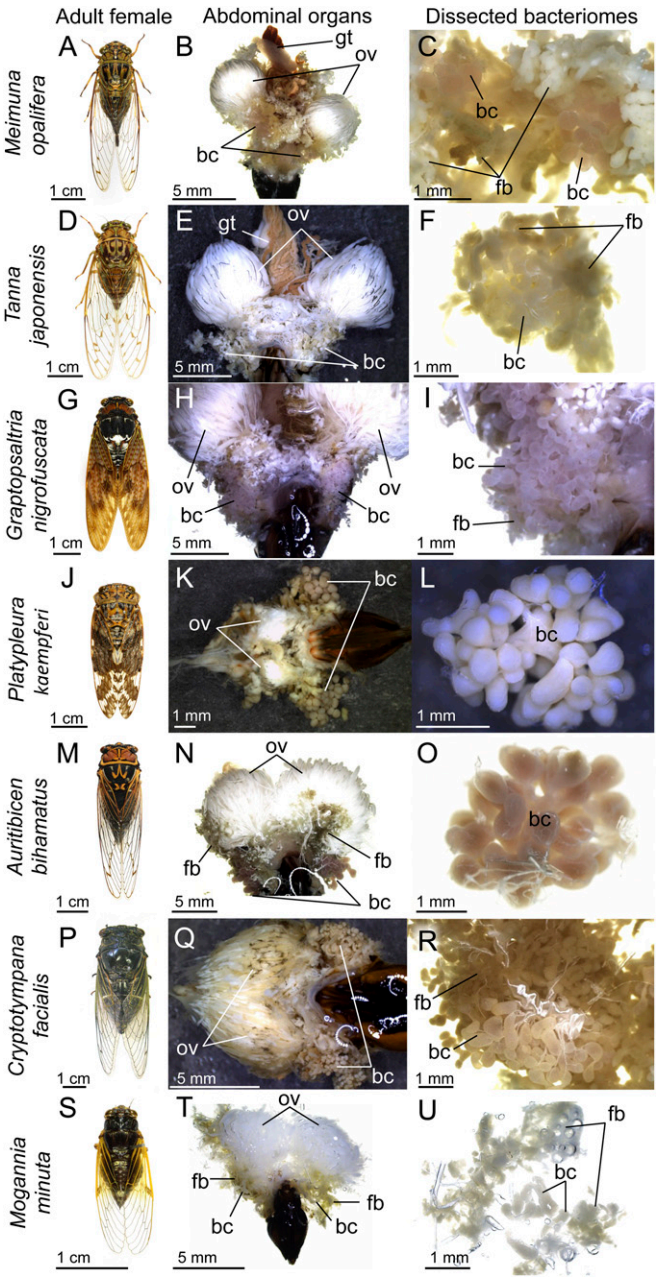

Fig. 1. Dissected symbiotic organs of cicadas. (*A–C*) *Me. opalifera*. (*D–F*) *Tanna japonensis*. (*G–I*) *G. nigrofuscata*. (*J–L*) *P. kaempferi*. (*M–O*) *Auritibicen bihamatus*. (*P–R*) *C. facialis*. (*S–U*) *Mo. minuta*. (*Left*) Photographs of adult females. (*Center*) Photographs show dissected abdominal organs. (*Right*) Photographs are enlarged images of dissected bacteriomes. bc, bacteriome; fb, fat body; gt, gut; ov, ovary.

While a substantial proportion of metagenomic reads and scaffolds corresponded to the nuclear genomes of the host cicadas, these data were not analyzed further because of the very low genomic coverage. We consistently identified 16S rRNA gene sequences of *Sulcia* from all 24 Japanese cicada species, which were phylogenetically placed in the cluster of cicada-associated *Sulcia* symbionts in the Flavobacteriaceae (*SI Appendix*, Fig. S2). On the other hand, although previous studies had identified *Hodgkinia* as another bacteriome symbiont in North American, South American, and Australian cicadas (47–53), our extensive PCR and metagenomic surveys detected *Hodgkinia* from only nine of 24 Japanese cicada species: three *Platypleura* species, three *Auritibicen* species, *Kosemia yezoensis*, *Vagitanus terminalis*, and *Muda kuroiwae* (*SI Appendix*, Fig. S3 and Tables S1, S2, and S5). In some cicada species, secondary bacterial symbionts, including *Wolbachia*, *Arsenophonus*, *Sodalis*, and *Spiroplasma*, were also detected (*SI Appendix*, Fig. S1 and Tables S1 and S2).

Genomics of *Sulcia* and *Hodgkinia* of Japanese Cicadas. All 20 *Sulcia* genomes determined by metagenomic sequencing were of the expected size, ranging from 0.24 to 0.28 Mb; were mostly syntenic with previously published *Sulcia* genomes; and encoded a set of bacterial genes similar to those identified in previously reported *Sulcia* genomes, which included most genes needed for synthesizing essential amino acids (*SI Appendix*, Fig. S4 and Table S4). The phylogeny of *Sulcia* genome sequences was highly congruent with the phylogeny of host mitochondrial genome sequences (*SI Appendix*, Fig. S5), confirming the expected codiversification between *Sulcia* and host cicadas over evolutionary time (13, 14). On the other hand, in all of the six *Hodgkinia*-associated Japanese cicada species subjected to metagenomic sequencing, the *Hodgkinia*-derived genomic contigs were never fully assembled, and their size, organization, guanine-cytosine (GC) content, and coverage variability suggested their origins from different *Hodgkinia* genomes coexisting in the same insect (*SI Appendix*, Figs. S1 and S6 and Table S5), as observed in some American cicadas (50–53). In all six cases, the total size of the identified *Hodgkinia* genomic contigs was greater, and much greater in some cases, than the size of the nonfragmented *Hodgkinia* genome identified from the North American cicada *Diceroprocta semicincta* (48) (*SI Appendix*, Table S5). In five of the six species, we identified two or more distinct copies of 16S rRNA genes, and in three of the six species, we identified multiple copies of a conserved *Hodgkinia* protein-coding gene, *rpoB* (*SI Appendix*, Fig. S6), as observed in the South American cicada genus *Tettigades* (50, 52). These observations strongly suggested that the *Hodgkinia* genomes are also fragmented and degenerated in Japanese cicadas. These *Hodgkinia* genomes were left as draft genome assemblies due to their complexity.

Conserved *Sulcia* and Frequent Lack of *Hodgkinia* in Japanese Cicadas. These results uncovered that while the ancient bacteriome symbiont *Sulcia* is highly conserved, the bacteriome cosymbiont *Hodgkinia* was missing in the majority of the Japanese cicada species. This finding was striking in that the *Hodgkinia* genome encodes biosynthetic pathways for several essential nutrients, including histidine, methionine, cobalamin, and riboflavin, which

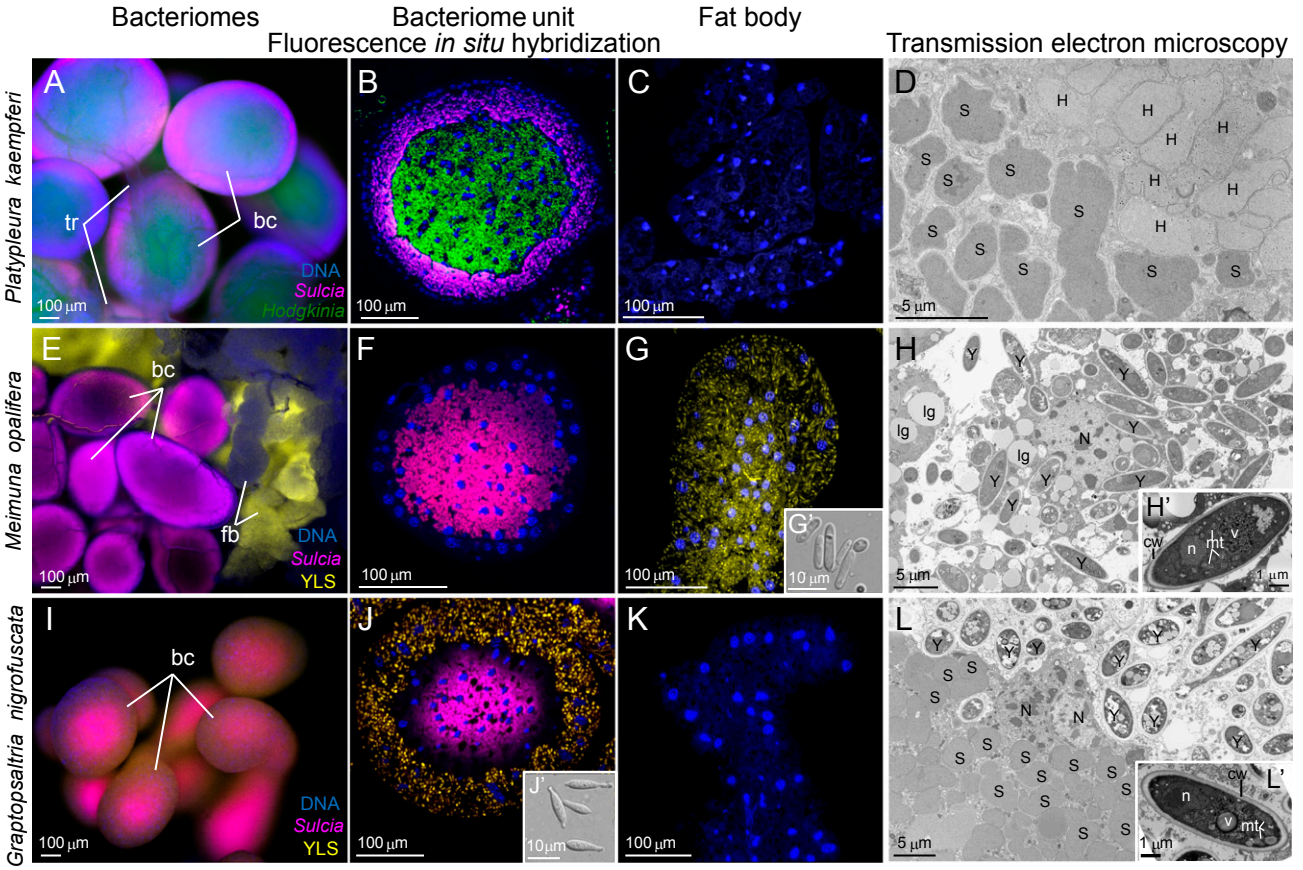

Fig. 2. In vivo localization and fine structure of *Sulcia*, *Hodgkinia*, and yeast-like fungal symbiont (YLS) of cicadas. (*A–D*) *P. kaempferi*. (*E–H*) *Me. opalifera*. (*I–L*) *G. nigrofuscata*. (*A*, *E*, and *I*) Whole-mount in situ hybridization of dissected bacteriomes. (*B*, *F*, and *J*) In situ hybridization of Technovit thin sections of bacteriome units. (*C*, *G*, and *K*) In situ hybridization of Technovit thin sections of fat body cells. Blue, magenta, green, and yellow visualize DNA, *Sulcia*, *Hodgkinia*, and YLS, respectively. *Insets* (*G′ and J′*) are enlarged light microscopic images of YLS cells. bc, bacteriome; fb, fat body; tr, trachea. (*D, H,* and *L*) Transmission electron microscopic images of the microbial symbionts. *Insets* (*H′* and *L′*) are enlarged images of YLS cells. cw, cell wall of YLS; H, *Hodgkinia*; lg, lipid granule; mt, mitochondrion of YLS; N, nucleus of host insect; n, nucleus of YLS; S, *Sulcia*; v, vacuole of YLS; Y, YLS.

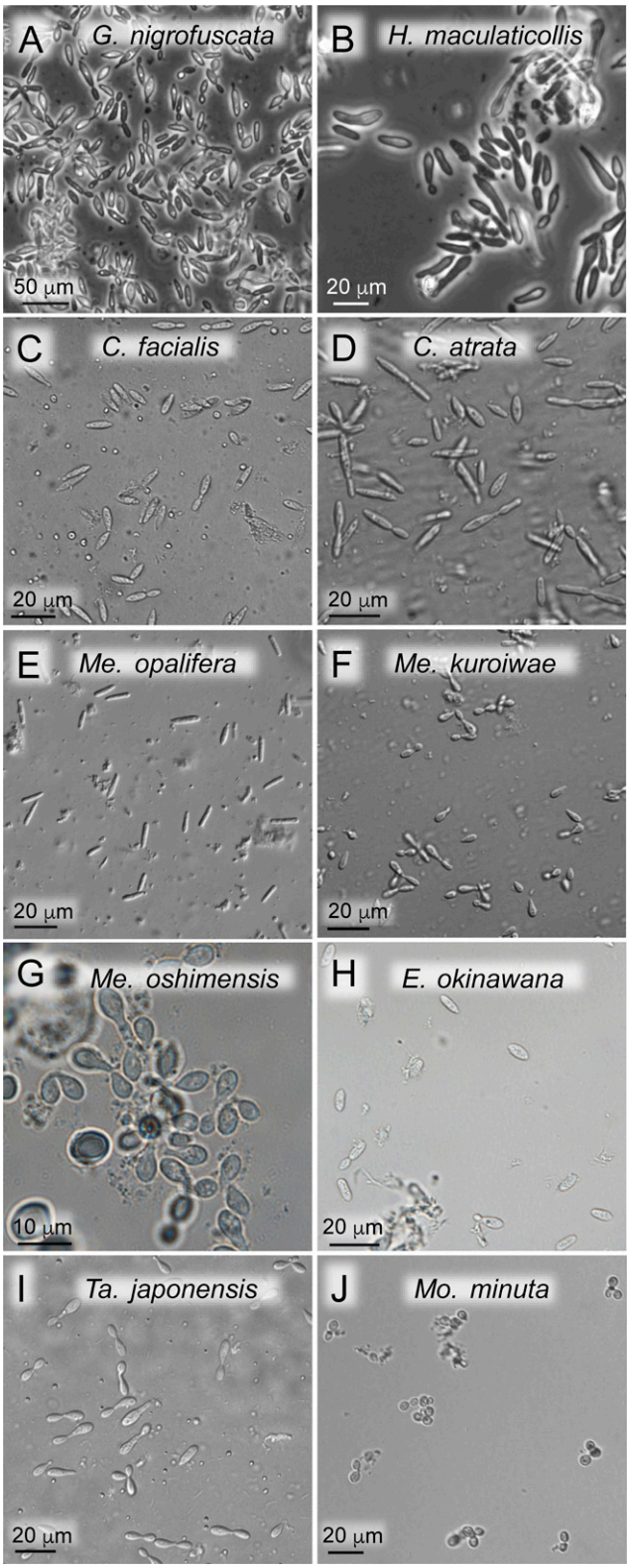

Fig. 3. Light microscopic images of yeast-like symbiont cells released from dissected cicadas. (*A*) *G. nigrofuscata*. (*B*) *H. maculaticollis*. (*C*) *C. facialis*. (*D*) *Cryptotympana atrata*. (*E*) *Me. opalifera*. (*F*) *Meimuna kuroiwae*. (*G*) *Meimuna oshimensis*. (*H*) *Euterpnosia okinawana*. (*I*) *Tanna japonensis*. (*J*) *Mo. minuta*.

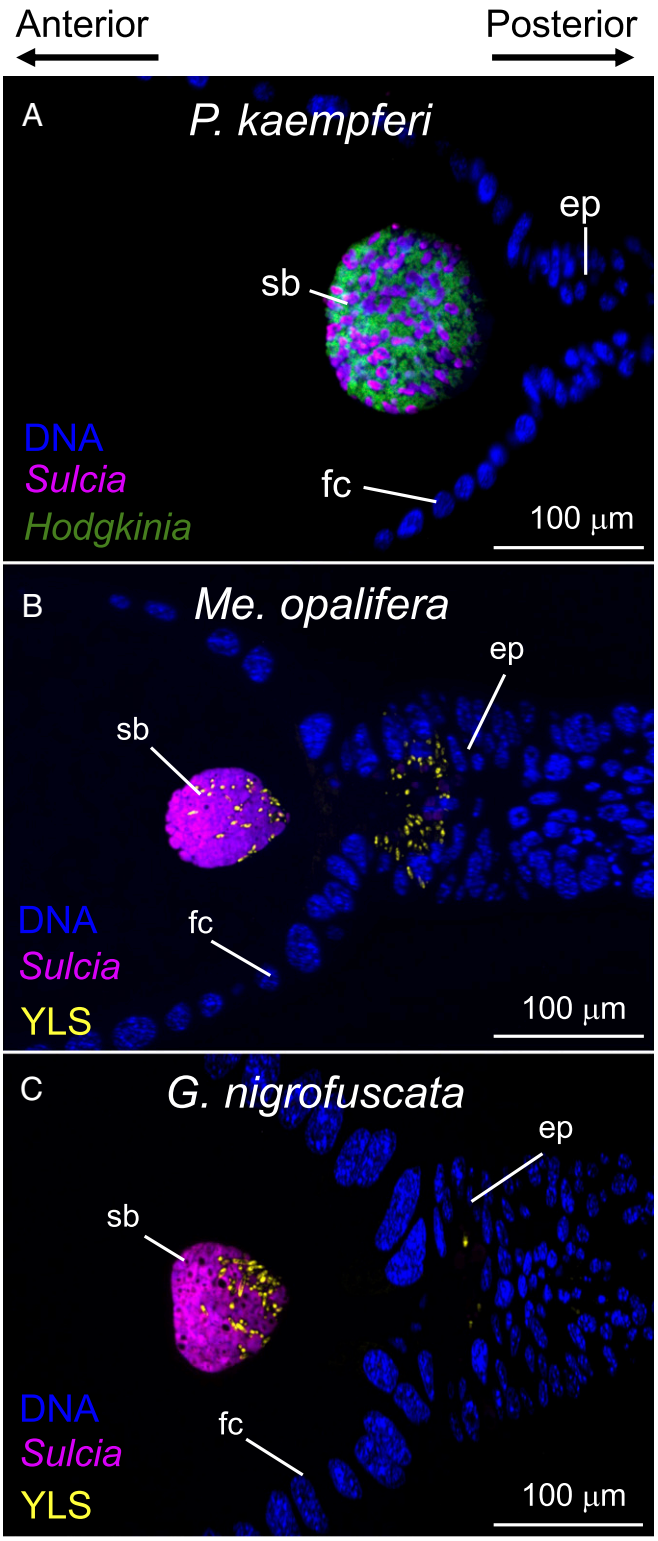

Fig. 4. Localization of *Sulcia*, *Hodgkinia* and yeast-like fungal symbiont at the posterior pole of developing oocytes of cicadas visualized by in situ hybridization. (*A*) *P. kaempferi*. (*B*) *Me. opalifera*. (*C*) *G. nigrofuscata*. Blue, magenta, green, and yellow indicate DNA, *Sulcia*, *Hodgkinia*, and yeast-like symbiont (YLS), respectively. In *B* and *C*, YLS cells are seen in the symbiont ball and also in the epithelial plug, which YLS was reported to infect for vertical transmission in planthoppers (78). ep, epithelial plug; fc, follicle cell; sb, symbiont ball.

are absent from the *Sulcia* genome, and thus the metabolic complementarity between *Sulcia* and *Hodgkinia* has been presumed to be important for survival of the cicadas feeding solely

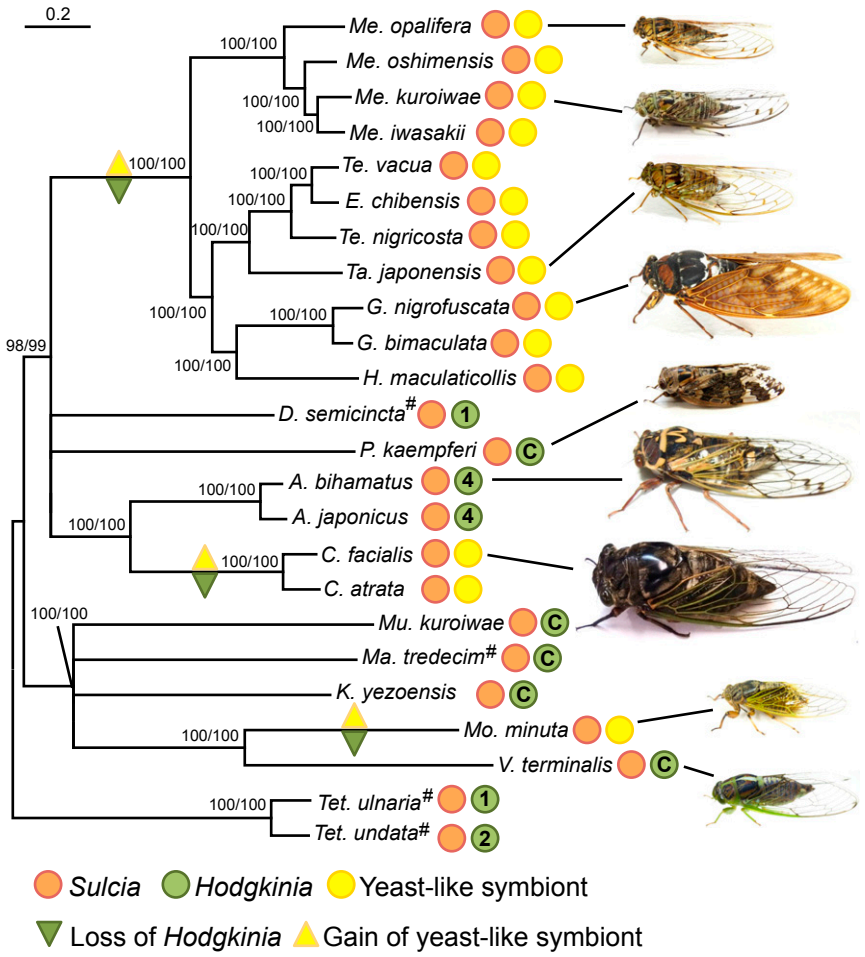

Fig. 5. Phylogenetic relationship of cicadas and their infection status with microbial symbionts. A maximum-likelihood phylogeny inferred from 15 mitochondrial gene sequences and 22 tRNAs (14,733 aligned nucleotide sites) of 20 Japanese cicada species, together with four previously studied American species (highlighted by #), is shown. Bootstrap support values are indicated on each node in the order of maximum-likelihood/neighbor-joining. Detected microbial symbionts are mapped on the right side of each species name with orange circles for *Sulcia*, green circles for *Hodgkinia*, and yellow circles for the yeast-like fungal symbiont. In the green circles of *Hodgkinia*, the number 1, 2, or 4 indicates the number of distinct *Hodgkinia* genomes that form a complex. C indicates highly fragmented *Hodgkinia* complexes in which the exact number of genomes could not be determined (51, 53). Colored triangles on the phylogeny indicate the estimated replacement events from *Hodgkinia* to the fungal symbionts. Selected images of adult cicadas are depicted to the right of the maximum-likelihood phylogeny. *A. bihamatus*, *Auritibicen bihamatus*; *C. atrata*, *Cryptotympana atrata*; *E. chibensis*, *Euterpnosia chibensis*; *G. bimaculata*, *Graptopsaltria bimaculata*; *Ma. tredecim*, *Magicicada tredecim*; *Me. iwasakii*, *Meimuna iwasakii*; *Me. oshimensis*, *Meimuna oshimensis*; *Ta. japonensis*, *Tanna japonensis*; *Te. nigricosta*, *Terpnosia nigricosta*; *Te. vacua*, *Terpnosia vacua*; *Tet. ulnaria*, *Tettigades ulnaria*; *Tet. undata*, *Tettigades undata*.

on nutritionally deficient plant xylem fluid (48, 49). How are these cicadas capable of surviving without *Hodgkinia*? In an attempt to address this question, we carefully inspected the Japanese cicadas morphologically, histologically, and cytologically.

Detection of Vertically Transmitted Fungal Symbionts in Cicadas Lacking *Hodgkinia*. In the cicada species associated with both *Sulcia* and *Hodgkinia*, such as *Platypleura kaempferi* and *Auritibicen japonicus*, each bacteriome unit consisted of three cellular components: surface sheath cells constituting the outermost epithelial cell layer to encase the whole bacteriome unit, peripheral bacteriocytes comprising the surface layer beneath the sheath cells, and a central syncytial cytoplasm located at the center of the bacteriome unit (*SI Appendix*, Fig. S7 *A* and *B* and Table S6). Light microscopy, fluorescence in situ hybridization targeting bacterial 16S rRNA, and transmission electron microscopy identified *Sulcia* in the peripheral bacteriocytes and *Hodgkinia* in the central syncytial cytoplasm, respectively (Fig. 2 *A–D* and *SI Appendix*, Fig. S8 *A* and *B*). On the other hand, in the cicada species associated with *Sulcia* only, such as *Meimuna opalifera*, *Graptopsaltria nigrofuscata*, *Cryptotympana facialis*, *Hyalessa maculaticollis*, and *Mogannia minuta*, while the surface sheath cells were clearly recognizable, the peripheral bacteriocytes and the central cytoplasm were indiscernible and comprised the inner bacteriome region (*SI Appendix*, Fig. S7 *B–E* and Table S6), where *Sulcia* was specifically localized (Fig. 2 *E*, *F*, *I*, and *J* and *SI Appendix*, Fig. S8 *C*, *F*, and *I*). Notably, when these cicada samples were dissected, numerous yeast-like budding particles were observed under the light microscope (Fig. 3). PCR amplification and sequencing identified fungal 18S rRNA gene sequences from these cicada species (*SI Appendix*, Table S1), which exhibited the highest similarities to 18S rRNA gene sequences of entomoparasitic fungi of the genus *Ophiocordyceps*, including *Ophiocordyceps longissima* (KJ878925), *Ophiocordyceps sobolifera* (EF468972), and *Ophiocordyceps yakusimensis* (AB044632). Reexamination of the Illumina reads confirmed the presence of fungal gene assemblies (*SI Appendix*, Fig. S1 and Table S2), although coverage values for the fungal assemblies were generally low, which was likely due to the low efficiency of DNA extraction from fungal cells with a thick cell wall. Fluorescence in situ hybridization targeting fungal 18S rRNA and transmission electron microscopy visualized the yeast-like symbionts in the fat body surrounding the bacteriomes (e.g., *Me. opalifera*, *C. facialis*, *Mo. minuta*) (Fig. 2 *G* and *H* and *SI Appendix*, Fig. S8 *D* and *J*), in the well-developed surface sheath cells (e.g., *G. nigrofuscata*) (Fig. 2 *I*, *J*, and *L*), or in both (e.g., *H. maculaticollis*) (*SI Appendix*, Fig. S8 *F* and *G*). Transmission electron microscopy confirmed that the fine structure of the yeast-like symbionts was typical of unicellular fungi with a nucleus, mitochondria, and thick cell wall (Fig. 2 *H* and *L*). Fluorescence in situ hybridization of ovaries dissected from adult females detected specific localization of not only *Sulcia* but also the yeast-like symbionts in developing oocytes, where the coinfecting symbionts formed a ball-shaped mass at the posterior pole (Fig. 4), indicating a vertical transmission route for the yeast-like symbiont that may be functionally equivalent to the transmission of *Sulcia* and *Hodgkinia*.

Recurrent Losses of *Hodgkinia* and Replacements by Fungal Symbionts. These results unveiled that while the ancient bacteriome symbiont *Sulcia* has been stably maintained in cicadas, the bacteriome cosymbiont *Hodgkinia* has not, which may be related

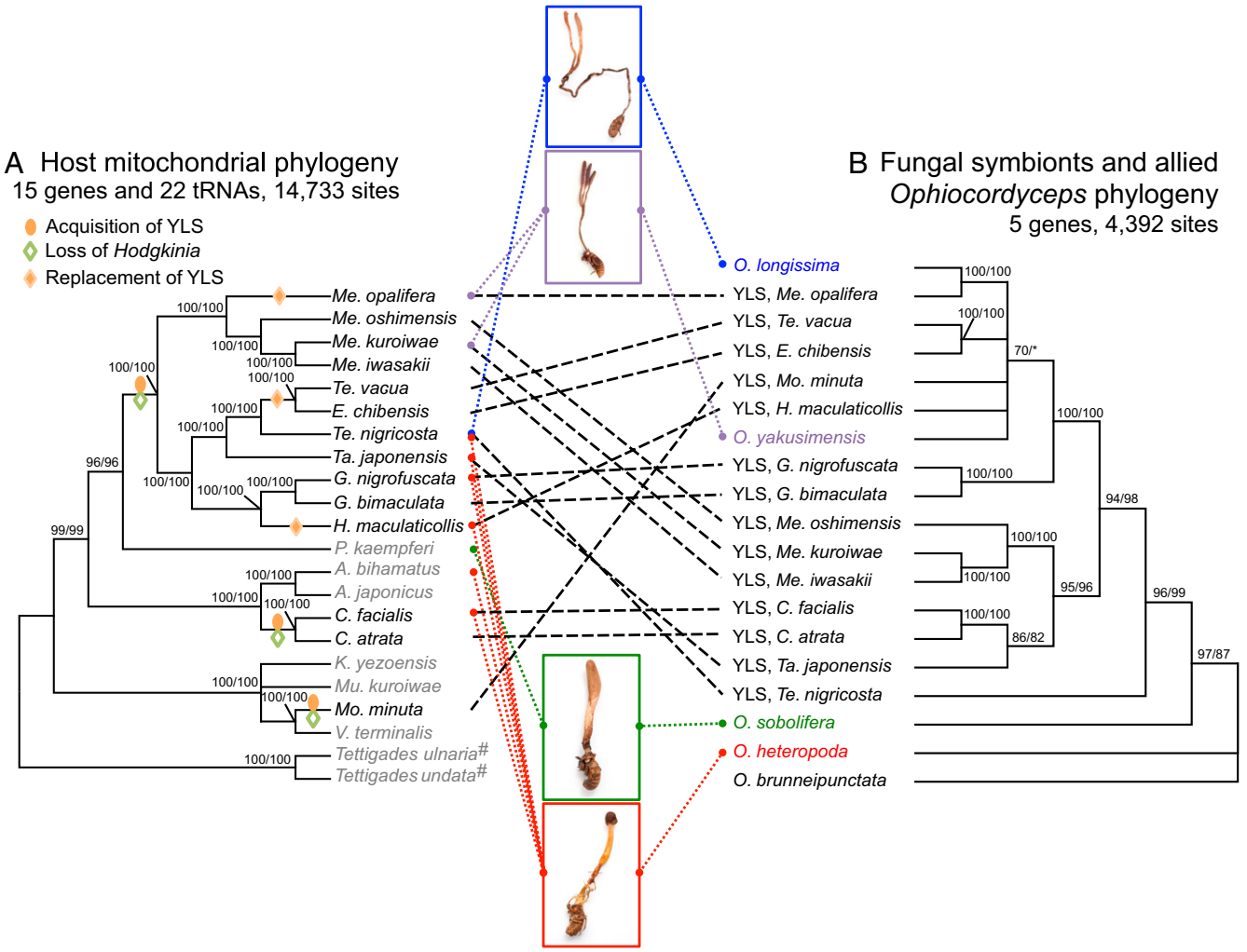

Fig. 6. Cophylogenetic analysis of host cicadas and their yeast-like fungal symbionts (YLS). (*A*) Maximum-likelihood phylogeny of 20 Japanese cicada species inferred from mitochondrial genome sequences (15 genes and 22 tRNAs, 14,733 aligned nucleotide sites), with two South American cicada species, *Tettigades* spp., as outgroup taxa. Fungus-associated cicada species are shown in black, whereas *Hodgkinia*-harboring cicada species are shown in gray. (*B*) Maximum-likelihood phylogeny of their fungal symbionts based on five nuclear gene sequences (4,392 aligned nucleotide sites), with allied *Ophiocordyceps* entomopathogenic fungi as ingroup and outgroup taxa. Host-symbiont connections are shown by black dashed lines. Cicada-parasitizing *Ophiocordyceps* fungi allied to the cicada symbionts, namely, *O. yakusimensis*, *O. longissima*, *O. sobolifera*, and *Ophiocordyceps heteropoda*, are highlighted by colors, and their host range records are shown by colored dotted lines according to ref. 57. Estimated replacement events from *Hodgkinia* to a fungus or from a fungus to another fungus are mapped on the phylogeny. *A. bihamatus*, *Auritibicen bihamatus*; *C. atrata*, *Cryptotympana atrata*; *E. chibensis*, *Euterpnosia chibensis*; *G. bimaculata*, *Graptopsaltria bimaculata*; *Me. iwasakii*, *Meimuna iwasakii*; *Me. kuroiwae*, *Meimuna kuroiwae*; *Me. oshimensis*, *Meimuna oshimensis*; *O. brunneipunctata*, *Ophiocordyceps brunneipunctata*; *Ta. japonensis*, *Tanna japonensis*; *Te. nigricosta*, *Terpnosia nigricosta*; *Te. vacua*, *Terpnosia vacua*.

to the extreme genome degeneration and fragmentation observed in some *Hodgkinia* lineages (50–53). On the grounds that the *Hodgkinia*-free cicada species always possess the fungal associates, the evolutionary process must have entailed replacement of *Hodgkinia* by the fungal symbiont. In this study, we found no cicada individuals containing both *Hodgkinia* and the fungal symbiont (*SI Appendix*, Table S1). According to the phylogeny of the Japanese cicadas based on mitochondrial genome sequences, on which their microbial symbionts were mapped, *Hodgkinia* has been replaced by the fungal symbiont repeatedly, at least three times and possibly more (Fig. 5).

Phylogenetic Placement and Diversity of Fungal Symbionts in Cicadas. Molecular phylogenetic analysis based on fungal 18S rRNA gene sequences showed that the fungal symbionts of cicadas formed a relatively well-supported clade within the genus *Ophiocordyceps* (*SI Appendix*, Fig. S9), an ascomycetous group consisting of entomopathogenic fungi with a number of cicada-parasitizing species (56, 57). This phylogenetic pattern highlighted a close evolutionary connection between the fungal symbionts of cicadas and the *Ophiocordyceps* entomopathogens. Furthermore, four additional fungal nuclear genes were amplified by PCR and sequenced for all of the 15 fungus-associated cicada species (*SI Appendix*, Table S1), which yielded a better resolved phylogenetic relationship of the fungal symbionts (*SI Appendix*, Fig. S10). Phylogenetic comparison of the host cicadas and the fungal symbionts (Fig. 6) showed that several cicada-parasitizing fungi, such as *O. longissima*, *O. yakusimensis*, and *O. sobolifera*, were placed within or just outside the clade of the cicada symbionts, favoring the hypothesis that the fungal symbionts of cicadas have evolved from cicada-parasitizing *Ophiocordyceps* fungi.

Phylogenetic comparison of the host cicadas and the fungal symbionts also showed that the phylogenetic relationship of the cicada symbionts was locally concordant with the phylogenetic lineages of the host cicadas, as exemplified by the fungal symbiont lineages associated with *Graptopsaltria* spp., *Cryptotympana* spp., and *Meimuna* spp. (except *Me. opalifera*), which was indicative of

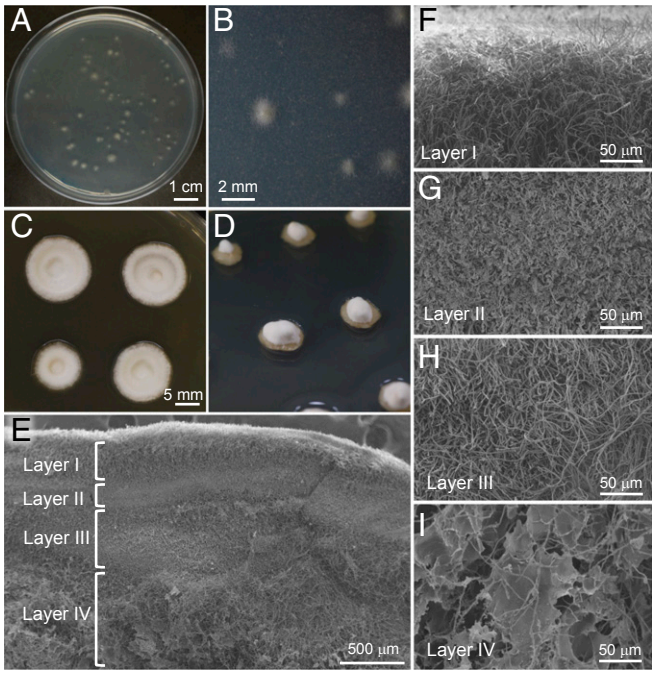

Fig. 7. Cultivated fungal symbiont strain from the cicada *Me. opalifera*. (*A*) Small colonies on a peptone-supplemented potato dextrose agar plate around 1 mo after inoculation. (*B*) Enlarged image of the colonies, which consist of radially arranged hyphae. (*C*) Large colonies about 3 mo after inoculation. Note that the colonies are thick and hard, constituting a dense mass of mycelia. (*D*) Mound-shaped colonies whose inoculum was a small piece of the dense mycelial mass cut from the precultured symbiont colony. Note that the colonies grow as hard and coherent fungal masses rather than stretching hyphae into flat colonies. (*E*) Scanning electron microscopic image of a cut plane of the mound-shaped colony, in which layered structures (layer I to layer IV from surface to inside) are observed. (*F*) Layer I, consisting of filamentous hyphae arranged outward like a rug surface. (*G*) Layer II, consisting of densely packed hyphae. (*H*) Layer III, consisting of entangled filamentous hyphae. (*I*) Layer IV, consisting of a matrix filling the space between relatively sparse filamentous hyphae.

some degree of host specificity, stable vertical transmission, and host-symbiont codiversification.

Globally, however, the phylogeny of the fungal symbionts was incongruent with the phylogeny of the host cicadas, reflecting dynamic evolutionary trajectories of the fungal symbionts over deeper evolutionary time, presumably involving repeated acquisitions, losses, and replacements. In Fig. 6, three replacement events from *Hodgkinia* to a fungus and three replacement events from a fungus to another fungus are estimated and mapped. It should be noted, however, that this estimate is parsimonious and minimal in number and that the actual evolutionary process may be more complex and dynamic. It should also be kept in mind that although seemingly less likely, the possibility of evolutionary reversals from a symbiotic to parasitic lifestyle cannot be excluded.

Recurrent Evolution of Fungal Symbionts from Parasitic Fungi in Cicadas. These results strongly suggest that the fungal symbionts of cicadas have repeatedly evolved from cicada-parasitizing *Ophiocordyceps* fungi, highlighting a straightforward connection between parasitism and symbiosis. It seems plausible, although speculative, that the ecological overlap between the cicada nymphs and the *Ophiocordyceps* entomopathogens in the plant rhizosphere (58), in combination with the ability of the *Ophiocordyceps*-allied entomopathogens to evade the insect immunity and survive and proliferate inside the insect body cavity (59–61), might have predisposed the recurrent evolution of the fungal symbionts from the fungal parasites in cicadas. In this context, it is notable that yeast-like fungal symbionts have been reported from diverse insect groups (1, 62–64), and some of them were identified to be phylogenetically allied to *Ophiocordyceps* entomopathogens as in aphids (25, 65, 66), scale insects (29), planthoppers (38, 39, 67–69), and leafhoppers (33, 35, 36, 70), suggesting the possibility that the *Ophiocordyceps* entomopathogens might be serving as an environmental source for the evolution of novel fungal symbionts in diverse insects.

Cultivation of Fungal Symbiont of Cicadas. We attempted to cultivate the fungal symbionts from the fungus-associated Japanese cicadas representing 6 species, 11 populations, and 53 individuals on standard nutrient agar media (*SI Appendix*, Table S7). From most of the samples, no growing *Ophiocordyceps* fungi were obtained, except for occasional fungal contaminants that were verified with 18S rRNA gene/internal transcribed spacer (ITS) region sequencing. Notably, numerous fungal colonies of uniform morphotype were reproducibly isolated only from *Me. opalifera* (Fig. 7 and *SI Appendix*, Table S7). Three fungal strains isolated from adult cicadas collected at three distinct localities in Japan yielded almost identical 18S rRNA gene sequences to each other and also to the fungal symbiont sequences derived from dissected bacteriomes of *Me. opalifera* (*SI Appendix*, Fig. S9), indicating that the fungal symbiont of *Me. opalifera* is cultivable. The symbiont cultivability in *Me. opalifera* may reflect the recent acquisition of the fungal symbiont in the host lineage, which is closely related to the cicada-parasitizing fungus *O. longissima* and derived from an allied cicada parasite (Fig. 6 and *SI Appendix*, Figs. S9 and S10). The cultivated strains of the fungal symbiont grew slower than the contaminant fungi that quickly grew hyphae and formed large colonies. After saline-suspended symbiont cells from adult *Me. opalifera* were spread on agar media, it took as long as over a month at 25 °C, or 2–3 wk at 28 °C, to form small colonies of several millimeters in diameter consisting of radial hyphae (Fig. 7 *A* and *B*). Subsequently, the colonies became thicker and mound-shaped, rather than spreading flat, thereby constituting a dense, thick, and hard mycelial mass with a layered structure, which looked like the fungal sclerotium (Fig. 7 *C*–*I*). It is notable that upon infection and killing of their insect host, *Ophiocordyceps* entomopathogens fill up the host body with a hardened mycelial mass called the sclerotium, and finally form fruiting bodies to produce ascospores and/or conidia (56, 57). The cultivable fungal symbiont of *Me. opalifera*, which exhibits prevalent infection in host populations (*SI Appendix*, Tables S1 and S7) and vertical transmission to developing oocytes (Fig. 4*B*), seems like an intermediate status between the free-living *Ophiocordyceps* entomopathogens and the uncultivable fungal symbionts associated with other cicada lineages. It may provide a promising model system for gaining insights into how the evolutionary transition from free-living through cultivable to uncultivable fungal associates has proceeded, as recently highlighted in gut bacterial symbioses in stinkbugs (71–74). Whether the cultivable fungal symbiont is detectable, existing, and surviving in the habitats of *Me. opalifera* is of ecological interest and deserving of future field surveys.

Genomic Features of Fungal Symbiont: Insight into Metabolic Complementarity and Symbiont Replacement. The fungal symbiont of *Me. opalifera* was grown in liquid culture for preparation of genomic DNA of sufficient purity and quantity suitable for PacBio single-molecule genome sequencing. Sequencing on four single-molecule real-time (SMRT) cells resulted in 186-fold coverage of a draft genome, which was 25.1 Mb in size and assembled into 32 contigs. Subsequent analyses revealed a highly compact genome with a 60.4% GC content and ∼7,000 protein-coding genes (0.278 genes per kilobase) with a median gene length of 1,580 bp (median exon size of 316 bp, median intron size of 57 bp). Repetitive DNA sequences made up only 9.55% of the assembled length, the majority of which were simple repeats and LTR elements (4.04% and 3.88%, respectively). We identified 14 full-length ribosomal RNA operons as well as a single mitochondrial genome contig of 170 kb in size (*SI Appendix*, Table S8). The fungal symbiont genome of *Me. opalifera* retained all

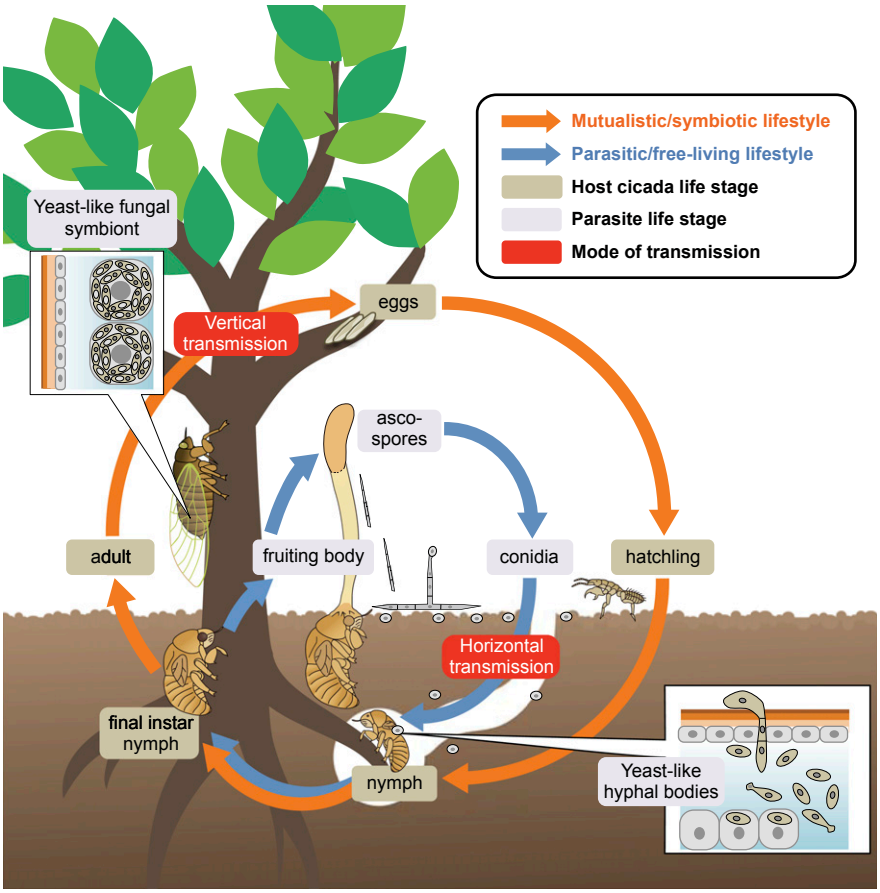

Fig. 8. Schematic illustration of the hypothetical ecological and evolutionary scenario as to how the fungal symbionts have been recruited from fungal parasites in cicadas.

synthesis pathway genes for essential and nonessential amino acids, B vitamins, and nitrogen recycling, which included the synthesis pathway genes for histidine and methionine that are provisioned by *Hodgkinia* (*SI Appendix*, Fig. S11). These results highlight metabolic versatility of the fungal symbiont that is more than sufficient to compensate for the absence of *Hodgkinia*. Genome sequencing and comparative genomics of the fungal symbionts of other cicada lineages will provide further insights into the processes and mechanisms of the dramatic symbiont replacements in cicadas.

Ecological and Evolutionary Connection of Fungal Symbionts and Parasitic Fungi. With all these results taken together, we propose a hypothetical perspective as to how the fungal symbionts of cicadas and the cicada-parasitizing *Ophiocordyceps* entomopathogens are interconnected to each other ecologically and evolutionarily in the natural environment (Fig. 8). Cicada nymphs spend many years in the soil of the plant rhizosphere, where they feed solely on xylem fluid from plant roots (55, 75). The ecology of cicada nymphs with constant and long-lasting exposure to humid and microbe-rich soil seems to facilitate contact and infection with pathogenic microorganisms. Notably, cicada parasites occupy a substantial fraction of the diversity of *Ophiocordyceps*-allied entomopathogens: For example, of some 240 species described from Japan, over 30 species (~13%) were reported to exploit cicadas (57). Hence, it is expected that cicada nymphs are frequently and constantly challenged by such fungal parasites in the natural environment. Upon invasion into the body cavity and before killing their insect host, *Ophiocordyceps*-allied entomopathogens proliferate in the hemolymph as nonhyphal yeast-like cells called hyphal bodies or blastospores (61, 76, 77). Because the morphology and localization of the hyphal bodies are quite reminiscent of those of the fungal symbionts, although speculative, we suggest the possibility that the yeast-like fungal symbionts may be derived from, and evolutionarily homologous to, the hyphal bodies of the fungal parasites. Once some variants/mutants of the fungal parasites attenuate virulence and become benign, it is expected that such fungal mutants may establish nonlethal and chronic infection within the host body in the form of hyphal bodies, instead of killing the host and developing fruiting bodies. However, it should be noted that evolving the mechanisms for getting entry into developing oocytes in the maternal body may be a substantial obstacle to establishing a transgenerational association via vertical transmission (78, 79). Considering the general metabolic versatility of fungi capable of synthesizing amino acids, vitamins, and other nutrients, such chronic fungal infections may entail a fitness benefit, especially in cicada lineages whose *Hodgkinia* has suffered massive genome degeneration. Furthermore, such fungal infections may additionally entail nonnutritional fitness benefits for the host cicadas, like conferring resistance to further microbial infections (80–82). Presumably, such fungal infections, establishments, and replacements are ongoing in the plant rhizosphere, which may have driven the recurrent evolution of the fungal symbionts in place of the ancient bacterial symbiont lineage. The ecological and evolutionary connection of the fungal symbionts to the fungal parasites in cicadas provides an impressive example of the parasitism-mutualism evolutionary continuum that has been advocated theoretically (83–87). In this context, the possibility that some fungal symbionts might exhibit a dual transmission strategy, in which vertical transmission to eggs in reproducing females coexists with host killing and spore formation for horizontal transmission in post-reproduction females and/or males, is theoretically predicted, whose verification deserves future studies.

On Diversity of Cicadas, *Ophiocordyceps* Entomopathogens, and Fungal Symbionts. Thus far, over 3,000 species of cicadas and some 500 species of *Ophiocordyceps*-allied entomopathogenic fungi have been described (54, 57). However, the taxonomy and systematics of

these groups are far from complete, and a large number of species are still waiting for discovery and description. Both cicadas and *Ophiocordiceps* fungi are the most diversified in warm and humid tropical/subtropical regions in the world, where the biodiversity is enormous, but thorough surveys are limited (54, 57). Recent studies on microbial symbionts of cicadas have identified the bacterial symbionts *Sulcia* and *Hodgkinia* but failed to detect the fungal symbionts (13, 47, 48, 50–53, 88). We expect that future studies on the diversity of tropical cicadas will uncover many more dynamic aspects of the evolution of microbial symbionts, plausibly involving numerous acquisitions, losses, and replacements across bacterial and fungal associates. Considering the metabolic versatility of the fungal symbiont relative to bacterial symbionts, fungal replacement of both *Sulcia* and *Hodgkinia* might also be possible in cicadas, as reported in some planthoppers and leafhoppers, wherein *Sulcia* and other ancient bacterial symbionts have been completely lost and taken over by fungal associates (35, 38). In the classic extensive histological surveys by German microbiologists (1, 44, 45, 89, 90), a comprehensive study detected fungal symbionts in as many as 237 (64%) of 370 species of plant-sucking hemipteran insects representing the Auchenorrhyncha (cicadas, spittlebugs, leafhoppers, treehoppers, planthoppers, etc.) (44), and recent studies, including this study, have shown that some of them are *Ophiocordyceps*-allied fungal symbionts (33, 35, 37–39, 67, 70). Here, we point out that such dynamic symbiont recruitment from fungal parasites may be taking place in diverse insects more generally than previously envisaged.

Concluding Remark. In North America, cicadas are well known by the general public for their relatively large size, their loud and musical songs, and their massive periodical emergence from the underground (75). In Asia, people recognized that bizarre-shaped mushrooms sometimes grow out of cicada nymphs and other insects underground, developing the mystic notion of animal/plant transformation and utilizing the insect/fungus complex for traditional medicinal purposes (91, 92). In Europe, early microbiologists microscopically described the universal occurrence of not only bacterial symbionts but also yeast-like fungal symbionts in a variety of insects (1, 44, 45, 89, 90), although their microbiological identity has long been elusive due to their fastidious nature and the lack of molecular tools at that time. Mycologists have described *Ophiocordyceps* and allied fungi as insect parasites, including many cicada-parasitizing species (56, 93–96). Recent molecular phylogenetic approaches have identified some of the yeast-like fungal symbionts of insects as close relatives of the *Ophiocordyceps* entomopathogens (25, 33, 35, 37–39, 65–70). Recent genomic approaches to the insect-associated microbial communities have uncovered many striking cases of drastic size reduction and extreme metabolic streamlining in ancient bacterial symbiont genomes, some of which look like they are almost going beyond the limit of being able to sustain basic cellular functioning (19–22). Among them, the ancient bacterial symbiont of cicadas, *Hodgkinia*, represents a striking case: The genome is reduced to only a small percentage of the size of the *Escherichia coli* genome, encodes less than 200 genes, supplies only a few essential nutrients to the host cicada, and is often highly fragmented into a number of minicircles, which is indicative of genomic instability and possibly at the edge of extinction due to genome erosion (47, 48, 50–53). In this study, these divergent lines of previous knowledge on cicadas and their associated microbes across time, space, and scale are integrated into a coherent picture, which sheds light on the dynamic ecological and evolutionary aspects of endosymbiosis entailing continual birth, decline, collapse, and renewal of intimate host-symbiont associations.

Materials and Methods

Cicada samples used in this study are listed in *SI Appendix*, Table S1. PCR, cloning, and sequencing of bacterial and fungal genes from dissected cicada tissues were performed using the primers listed in *SI Appendix*, Table S9. Metagenomic libraries of dissected symbiotic organs were constructed using the TruSeq DNA PCR-Free Library Preparation kit or the NEBNext Ultra DNA Library Prep Kit, and sequenced on an Illumina HiSeq 2500 system. Quality-trimmed reads were assembled, and resultant contigs were annotated and visualized using custom Python and Processing scripts. Bacterial and fungal symbionts in cicada tissues and cells were visualized and observed by light microscopy, whole-mount fluorescence in situ hybridization, in situ hybridization of methacrylate resin thin sections, and transmission electron microscopy. In situ hybridization was performed using the fluorochrome-labeled probes listed in *SI Appendix*, Table S10. Fungal cultivation was conducted using nutrient agar media supplemented with antibiotics as detailed in *SI Appendix*, Table S7.

Complete details on the materials and methods are provided in *SI Appendix, SI Materials and Methods*.

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