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.

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–LC370730) and the GenBank database (accession nos. MG737715–MG737734, CP029009–CP029028, SAMN0893808–SAMN08938010, 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 non-adaptive 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 Ophiocordycipes fungi, identifying the evolutionary source of the fungal symbionts as the fungal parasites of cicadas. These results highlight a coevolutionary connection between parasitism and symbiosis, and unveil an evolutionary trajectory to compensate for a deteriorating ancient bacterial symbiont by domesticated endomycopathogens.

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).
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; and encoded a set of bacterial genes similar to those identified in previously published Hodgkinia genomes. We consistently identified 16S rRNA gene sequences of Hodgkinia from all 24 Japanese cicada species, which were phylogenetically placed in the cluster of cicada-associated Hodgkinia genomes. 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.

**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; and Frequent Lack of genomic variation 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 S3). 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 are necessary for the survival of the cicada host.
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. 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 kuroiwaite. (G) Meimuna oshimensis. (H) Euterpnesia 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.
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, Graptopsis nigrofusca, Cryptotympana facialis, Hyalessa maculatillicollis, 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 yaksimensis (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. nigrofusca) (Fig. 2 I, J, and L), or in both (e.g., H. maculatillicollis) (SI Appendix, Fig. S8 F and G). Transmission electron microscopy confirmed that the fine structure of the yeast-like symbions 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 symbions in developing oocytes, where the cofecting symbions formed a ball-shaped mass at the posterior pole (Fig. 4), indicating a vertical transmission route for the 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...
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. 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 rDNA 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.

**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*, *Auribacien bihamatus*; *C. atrata*, *Cryptotympana atrata*; *E. chibensis*, *Euterpnosia chibensis*; *G. bimaculata*, *Graptosaltria bimaculata*; *I. wasakii*, *Meimuna iwasakii*; *K. yezoensis*, *Meimuna kuroiwae*; *M. minuta*, *Meimuna opalifera*; *O. brunneipunctata*, *Ophiocordyceps brunneipunctata*; *T. japonensis*, *Tanna japonensis*; *T. nigricosta*, *Terpnosia nigricosta*; *T. vacua*, *Terpnosia vacua*.
some degree of host specificity, stable vertical transmission, and host-symbiont codiversification.

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), plant hoppers (38, 39, 67–69), and leaffoppers (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 congruent with 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. 4B), seems like an intermediate stage between the free-living Ophiocordyceps entomopathogens and the uncultivable fungal symbions associated with other cicada lineages. It may provide a promising model system for gaining insights into how the evolutionary transition from free-living to cultivable to uncultivable fungal associates has proceeded, as recently highlighted in gut bacterial symbioses in stickbugs (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
Thus far, over 3,000 species of cicadas and some 500 entomopathogenic fungi have been described (54, 57). However, the taxonomy and systematics 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 more than sufficient to compensate for the absence of Hodgkinia, whose verification deserves future studies.

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.

**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 more than sufficient to compensate for the absence of Hodgkinia, whose verification deserves future studies.

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.
these groups are far from complete, and a large number of species are still waiting for discovery and description. Both cicadas and *Ophiocordycip* fungi are the most diversified in warm and humid tropical/subtropical regions in the world, where the biodiversity is high but bombardments 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 associates, including the fungal replacement of both *Sulcia* and *Hodgkinia* might also be possible in cicadas, as reported in some plant hopplers and leaffeepers, 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 hemipterans representing the Auchenorrhincha (cicadas, spittlebugs, leaffeepers, plant hopplers, etc.) (44), and recent studies, including this study, have shown that some of them are *Ophiocordyci*-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 symbioses in some insects undergoing 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 *Ophiocordyces* 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 *Ophiocordyces* 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 NEBNext Ultra DNA Library Prep 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 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 fluorescently 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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1. Buchner P (1965) Endosymbiosis of Animals with Plant Microorganisms (Interscience, New York).

2. Boutziou K, Miller TA (2003) Insect Symbiosis (CRC, Boca Raton, FL).

3. Zchori-Fein E, Miller TA (2011) Manipulative Tenants: Bacteria Associated with Insects and Their 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 *Ophiocordyces* 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 *Ophiocordyces* entomopathogens (25, 33, 35, 37–39, 65–70). Recent genomic approaches to the insect-associated microorganisms 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.

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Complete details on the materials and methods are provided in SI Appendix, SI Materials and Methods.
26. Manzano-Marín A, Szabó G, Simon JC, Horn M, Latorre A (2017) Happens in the best of subfamilies and repeated replacements of co-obligate secondary endosymbionts within Lachninae aphids. Environ Microbiol 19:393–408.

27. Meseguer AS, et al. (2017) Buchnera has changed flatmate but the repeated replacement of co-obligate symbionts is not associated with the ecological expansions of its aphid host. Mol Ecol 26:3855–3868.

28. Chong RA, Moran NA (2018) Evolutionary loss and replacement of Buchnera, the obligate endosymbiont of aphids. ISME J 12:898–908.

29. Gomez-Polo P, et al. (2017) An exceptional family: Ophiorhizoides-allied fungus dominates the microflora of soft scale insects (Hemiptera: Sternorrhyncha: Coccidae). Mol Ecol 26:5855–5868.

30. Kobaika M, Michalka A, Walczak M, Junkert K, Szklarczewicz T (2016) Sulcia symbiont of the leafhopper Macrosteles ravis (Ribaut, 1927) (Insecta, Hemiptera, Cicadellidae: Deltocephalinae) harbors Arsenosporospora bacteria. Protoplasma 253:903–912.

31. Kobaika M, Michalka A, Walczak M, Szklarczewicz T (2018) Dual “bacterial/fungal” symbiosis in Deltocephalinae leafhoppers (Insecta, Hemiptera, Cicadomorpha: Cicadellidae). Microb Ecol 75:771–782.

32. Noda H, Nakashima N, Koizumi M (1995) Phylogenetic position of yeast-like symbionts in plant hopper: an insect model system for insect-microbe symbiotic associations. Res Microbiol 146:789–793.

33. Smith WA, et al. (2013) Phylogenetic analysis of symbionts in feather-feeding lice of the genus Columbicola: Evidence for repeated symbiont replacements. BMC Evol Biol 13:109.

34. Müller HI (1949) Zur systematik und phylogenie der zikaden-endosymbionten. Biol Zentr 68:343–368. German.

35. Müller HI (1962) Neue Entwicklungen über verbreitung und phylogenie der endosymbionten zikaden. Z Morphol Ökol Tiere 51:190–210. German.

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

37. McCutcheon JP, McDonald BR, Moran NA (2008) Emergence of evolutionary superiority in a bacteriome and alternative genetic code in the extremely small and GC-rich genome of a bacterial symbiont. PLoS Genet 5: e1000565.

38. McCutcheon JP, McDonald BR, Moran NA (2009) Convergent evolution of metabolic roles in bacterial endosymbionts of insects. Proc Natl Acad Sci USA 106:15384–15393.

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

40. Van Leuven JT, Meier RC, Simon C, McCutcheon JP (2014) Sympatic speciation in a bacterium: symbiont results in two genomes with the functionality of one. Cell 158:1270–1280.

41. Campbell MA, et al. (2015) Genome expansion via lineage splitting and genome reduction in the cicada endosymbiont Hodgkinia. Proc Natl Acad Sci USA 112:10192–10198.

42. Lukasik P, et al. (2018) Multiple origins of interdependent endosymbiotic complexes in a genus of cicadas. Proc Natl Acad Sci USA 115:E226–E235.

43. Campbell MA, Lukasik P, Simon C, McCutcheon JP (2017) Idiosyncratic genome degradation in the cicada endosymbiont of periodical cicadas. Curr Biol 27:3568–3575.e3.

44. Sanborn AF (2014) Catalogue of the Cicadidae (Hemiptera: Auchenorrhyncha) (Academ, New York).

45. Hayashi M, Saisyo Y (2011) The Cicadidae of Japan (Seibundo-Shinkosha, Tokyo).

46. Sugi GH, et al. (2007) Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. Stud Mycol 57:5–59.

47. Japanese Society of Cordyceps Research (2014) An Illustrated Guide to Ecology of Japanese Cordyceps (Seibundo-Shinkosha, Tokyo).

48. Nikoh N, Fukuta T (2009) Interkingdom host jumping underground: Phylogenetic analysis of entomopathogenic fungi of the genus Cordyceps. Mol Biol Evol 17:629–638.

49. Vilinskas A, Götz P (1999) Parasitic fungi and their interactions with the insect immune system. Adv Parasitol 43:267–313.

50. Wang C, St Leger RJ (2000) A collagenous protective coat enables Metarhizium anisopliae to evade insect immune responses. Proc Natl Acad Sci USA 103:6647–6652.