

Insights into the Ecology and Evolution of Blattodea-associated *Ophiocordyceps*

Joao Araujo (✉ joaofungo@gmail.com)

University of Florida <https://orcid.org/0000-0003-2529-7691>

G.M. Moriguchi

Okinawa Daigaku

S. Uchiyama

Cordyceps Society for Cordyceps Research

N. Kinjo

The Japanese Society of Cordyceps Research

Yu Matsuura

University of the Ryukyus: Ryukyu Daigaku

Research

Keywords: Hypocreales; Cockroaches; Termites; Entomopathogens; Host-jump, *Ophiocordyceps salganeicola*

Posted Date: September 3rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-68793/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at IMA Fungus on February 5th, 2021. See the published version at <https://doi.org/10.1186/s43008-020-00053-9>.

Abstract

Entomopathogenic fungi are ubiquitous inhabitants of forests worldwide, remarkably in tropical regions. Among these fungi, one of the most abundant and diverse is the genus *Ophiocordyceps*. These fungi are particularly diverse and more commonly found parasitizing coleopteran, lepidopteran, hymenopteran and hemipteran insects. However, other insect orders are also parasitized by these fungi, for example the blattodeans (termites and cockroaches). Despite their ubiquity in nearly all environments insects occur, blattodeans are rarely found infected by filamentous fungi and thus, their ecology and evolutionary history remains obscure. In this study, we propose a new species of *Ophiocordyceps* infecting the social cockroaches *Salganea esakii* and *S. taiwanensis*, based on 16 years of collections and field observations, especially the Ryukyu Archipelago. We found a high degree of genetic similarity between specimens from different islands, infecting two *Salganea* species and that this relationship is ancient, likely not originated from a recent host jump. Furthermore, we found that *Ophiocordyceps* lineages infecting cockroaches evolved around the same time, at least twice, one from beetles and the other from termites. We have also investigated the evolutionary relationships between *Ophiocordyceps* and termites and present the phylogenetic placement of *O. blattae* for the first time.

Introduction

The genus *Ophiocordyceps* is composed almost entirely of insect parasites, with few examples of beneficial endosymbionts of sap-sucking hemipterans (Quandt et al. 2014, Gomez-Polo, et al. 2017, Matsuura et al. 2018). The genus was erected by Petch (1931) to accommodate species of *Cordyceps* exhibiting clavate asci containing spores that do not disarticulate into partspores, contrasting with “the majority of the species of *Cordyceps* which have been described” at that time, exhibiting cylindrical asci and spores that readily disarticulate into numerous short partspores upon maturity. The diversity of *Ophiocordyceps* has been increasingly unraveled in the last decade, especially with discoveries of species associated with Hymenoptera, Lepidoptera and Hemiptera (Araújo et al. 2015, Araújo et al. 2018, Luangsa-ard et al. 2018). However, our knowledge about species associated with blattodean insects (cockroaches and termites) is still restricted to relatively few species, especially regarding cockroach parasites. We currently only know 10 species infecting termites, i.e. *O. bispora* – (Stiffler 1941); *O. octospora* – (Blackwell and Gilbertson 1981); *O. communis* – (Sung et al. 2007); *O. asiatica*, *O. brunneirubra*, *O. khokpasiensis*, *O. mosingtoensis*, *O. pseudocommunis*, *O. pseudorhizoidea* and *O. termiticola* – (Tasanathai et al. 2019). Furthermore, there are only three species described infecting cockroaches, i.e. *O. blattae* from Sri Lanka – (Petch 1931); *O. blattarioides* from Colombia – (Sanjuán et al. 2015) and *O. salganeicola* sp. nov. from Japan – this study).

The cockroaches (Blattodea) are ubiquitous organisms occupying almost all habitats where insects occur (Schal et al. 1984). They play a key ecological role as decomposers, with many examples of species living within and feeding on rotting wood, including a lineage of social species that evolved into the termites (Bell et al. 2007, Maekawa et al. 2008, Bourguignon et al. 2018, Evangelista et al. 2019). The parasitism of microsporidians and protozoans on these insects is relatively common and known for more

than a century (Crawley 1905, Woolever 1966, Purrini et al. 1988). However, the cases of infection by filamentous fungi on cockroaches have been rarely reported.

Although examples of *Ophiocordyceps* parasitizing Blattodea are scarce, the type of the genus is *O. blattae* infecting *Blatta germanica* (see original illustration in Fig. 1, adapted from Petch 1934). It was originally collected only in two occasions in Hakgala, Sri Lanka (Ceylon back in that time) with recent few records from Thailand (Luangsa-ard et al. 2018). Another example of an *Ophiocordyceps* infecting a cockroach is *O. blattarioides* (= *Paraisaria blattarioides* – see Mongkolsamrit et al. 2019). This species was described from Colombia with records in Belize and tropical lowlands in the Amazonian side of Andes region of Ecuador (Sanjuán et al. 2015). Both *O. blattae* and *O. blattarioides* exhibit striking morphological and ecological differences. For example, *O. blattae* forms a cylindrical ascoma producing elongated-fusoid ascospores, which do not disarticulate into partspores, measuring 50–80 × 3–4 μm, while *O. blattarioides* exhibit a stalk bearing a globose fertile part at the tip, producing spores that disarticulate into partspores of 6–12 × 1.5 μm. The host death location is also distinct with *O. blattae* killing its hosts on the underside of leaves while *O. blattarioides* is found buried in the leaf litter. Generally speaking, there is not sufficient information on the evolution and ecology of cockroach-associated entomoparasitic fungi.

In this study, we aim to propose a new species of *Ophiocordyceps* that parasitizes two social wood-feeding cockroach species distributed in southwestern part and Nansei islands of Japan, both living inside decaying logs. We provide morphological, molecular and ecological data to support the new species proposal with insights into the evolutionary origins of the parasitic fungi of cockroaches and their close related termites. We also present, for the first time, the phylogenetic position of *O. blattae*.

Figure 1. *Ophiocordyceps blattae* illustrations. **a.** the holotype original illustration of the host with fungus arising laterally and ascus with septate ascospores (adapted from Petch 1924); **b.** Illustration of *O. blattae* from Thailand used in this study (the actual specimen), with two ascomata arising laterally on both sides.

Materials And Methods

Sampling

Surveys were undertaken in the Japanese warm temperate and subtropical evergreen forests mainly consisting of trees belonging to *Fagaceae*, *Lauraceae* and *Theaceae* in Kunigami-son, Okinawa, Yakushima, Kagoshima and Nobeoka, Miyazaki. The parasitized cockroach samples of two host species, namely *Salganea esakii* and *taiwanensis*, were mainly collected in the small humid valley or riparian forests where *Castanopsis sieboldii*, *Distylium racemosum* and *Schefflera heptaphylla* grow, but also in the secondary forest harboring *Alnus japonica* after the deforestation of *Castanopsis* in Okinawa. The specimens used in this study were always found hidden inside soft rotten logs or large fallen branches of *C. sieboldii*, *A. japonica*, and so forth, with only the fungus sprouting out (Fig. 2). The infected cockroaches, and the substrata they were attached to, were collected in plastic containers and

transported to the laboratory. Some specimens were investigated immediately after the collection, while others were dried for many years before analyzed. The specimens were photographed individually, using a Canon 7D camera, equipped with an EF-100 mm macro lens or a MP-E 65 mm (5X) lens with a MT-24EX Canon macro lite flash attached.

Fig. 2. *Ophiocordyceps salganeicola* emerging from *Salganea esakii* in its natural habitat in Yakushima, isl. Kagoshima. **A-B.** Visible part of the ascoma, erupting from a hole in the wood; **C-D.** Hosts buried in the wood, visible only after digging few centimeters; **E.** Infected host on its nymphal stage, very rare.

Morphological studies

For macro-morphological characterization, specimens were examined using a stereoscopic microscope (Leica S8 APO) and sorted for further micro-morphological investigation. The characters investigated were ascomatal size, colour, position, presence/absence and characterization of asexual morphs and perithecial insertion (e.g. immersed, semi-immersed, erumpent, superficial). For micro-morphological characterization, either free-hand or cryo-sectioning of the ascoma was performed using a Leica CM1850 Cryostat. Samples were mounted on a slide with plain lactic acid or lacto-fuchsin (0.1g of acid fuchsin in 100 mL of lactic acid) for light microscopy examination using an (Nikon Eclipse Ni-U). A minimum of 50 ascospores were measured for morphological comparison. The illustrations of fungal specimens were drawn based on the observation of photographs using drawing pens 0.13 mm and 0.2 mm (Rotring), painted by watercolors (HOLBEIN Art Materials Inc.) and scanned for imaging (Fig. 3). We also present the morphological comparison between *Ophiocordyceps* species infecting cockroaches and termites (Table 1).

Fig. 3. Illustrations of *Ophiocordyceps salganeicola* at various stages of development. **A.** an illustration of a teleomorph of *O. salganeicola* growing on *Salganea esakii* collected in Miyazaki on June 22, 2005. **B.** teleomorph roach on *S. esakii* collected in Yakushima on June 17, 2017 and a closeup illustration of perithecia. **C-E.** distinct developmental stages of *O. salganeicola* on *S. taiwanensis*, collected in Katsuradake, Nago-shi, Okinawa Apr 23, 2016 **D.** Mori04 and **E.** Kunigami-son, Okinawa on May 29, 2016.

DNA extraction, PCR and sequencing

All specimens used in this study were collected in their natural habitat. The material was preserved either dried or in ethanol and DNA extractions were performed with the following protocol: Parts of fungal tissues were removed from the host, placed in 1.5 ml Eppendorf tubes with 100-200 µl of CTAB readily after its collection and stored at a room temperature, or entire samples were immersed in 70% ethanol and stored in the freezer. For DNA extraction, the samples were ground mechanically, added 400 µl of CTAB and incubated at 60°C for 20 min and centrifuged for 10 min at 14,000 rpm. The supernatant (approx. 400 µl) was transferred to a new 1.5 ml Eppendorf tube, mixed with 500 µl of 24:1 Chloroform: Isoamyl-alcohol (Sigma) and mixed by inverting. The mix was then centrifuged for 20 min at 14,000 rpm and the supernatant transferred to a new 1.5 ml Eppendorf tube and further cleaned using the

GeneCleanIII kit (MP Biomedicals), following the recommended protocol. The only step modified was the addition 30 µl of GlassMilk per sample, instead of the recommended 10 µl, aiming to increase yield.

Five loci were used in the analyses, i.e. small subunit nuclear ribosomal DNA (SSU), large subunit nuclear ribosomal DNA (LSU), translation elongation factor 1- α (TEF) and the largest and second largest subunits of RNA polymerase II (RPB1 and RPB2 respectively) with a total read length of 4,815 bp. The primers used were, SSU: 82F (5'-GAAACTGCGAATGGCT-3') and 1067R (5'-TMTCGTAAGGTGCCGA-3') (Matsuura et al. 2018); LSU: LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAACTTCG-3') (Vilgalys and Sun, 1994); TEF: 983F (5'-GCYCCYGGHCA YCGTGAYTTYAT-3') and 2218R (5'-ATGACACCRACRGCACRGTGTG-3'); cRPB1: (5'-CCWGGYTTYATCAAGAARGT-3') and RPB1Cr (5'-CCNGCDATNCRTRTCCATRTA-3') (Castlebury et al. 2004). RPB2: fRPB2-5F:(5'-GAYGAYMGWGATCAYTTYGG-3') and fRPB2-7cR (5'-CCCATRGCTTGTYRCCCAT -3').

Each 20 µl-PCR reaction contained 10 µl of Ampdirect® Plus 2x (Shimazu Corp.), 0.6µl of each forward and reverse primers (10 mM), 1 µl of DNA template, 0.1 TaKaRa *ExTaq* DNA Polymerase (TaKaRa) and 8.7 µl of Ultra Pure Distilled Water (Gibco). The PCR reactions were placed in a Astec PC-818 thermocycler under the following conditions: for SSU and LSU (1) 2 min at 95 °C, (2) 10 cycles of denaturation at 95 °C for 30 sec, annealing at 62 °C for 30 sec, and extension at 72 °C for 2 min, followed by (3) 25 cycles of of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 2 min and (4) 3 min at 72 °C. For TEF and RPB1(1) 2 min at 95 °C, (2) 10 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 40 sec, and extension at 72 °C for 1 min 30 sec, followed by (3) 30 cycles of of denaturation at 95 °C for 30 s, annealing at 55 °C for 40 sec, and extension at 72 °C for 1 min 30 sec and (4) 3 min at 72 °C. Each PCR reaction was partially electrophoresed and the rest was cleaned by adding 3.0 µl of enzymatic PCR clean-up reagent, consisting of 0.1 µl of Exonuclease I (New England BioLabs) and 0.1 µl of alkaline phosphatase (shrimp) (TaKaRa), incubated at 37 °C for 20 min and 80 °C for 15 min in the thermocycler. The processed PCR products were directly sequenced by a capillary DNA sequencer, Genetic Analyzer 3130xl (Applied Biosystems) at C-RAC of the University of the Ryukyus.

Phylogenetic analyses

The raw sequence reads (.ab1 files) were edited manually using Geneious 11.1.5 (<https://www.geneious.com>). Individual gene alignments were generated by MAFFT (Kato & Stanley 2013). The alignment of every gene was improved manually, annotated and concatenated into a single combined dataset using Geneious 11.1.5 (<https://www.geneious.com>). Ambiguously aligned regions were excluded from phylogenetic analysis and gaps were treated as missing data. The final alignment length was 4,629 bp: 1,020 bp for SSU, 870 bp for LSU, 967 bp for TEF, 683 bp for RPB1 and 1,089 for RPB2. Maximum likelihood (ML) analysis was performed with RAxML version 8.2.4 (Stamatakis 2006) on a concatenated dataset containing all five genes. The dataset consisted of 11 data partitions, 2 for SSU and LSU, and 9 for each codon position of the three protein coding genes: TEF, RPB1 and RPB2. The GTRGAMMA model of nucleotide substitution was employed during the generation of 1,000 bootstrap replicates. The sequences for all *Ophiocordyceps* used in this study are presented in Table 2.

Results

Collections

We collected 26 samples in two locations in Okinawa, 27 in Yakushima isl., Kagoshima, and 5 in Miyazaki. Four specimens from Yonahadake and Kunigami (Okinawa) and one from Kagoshima were used for DNA extraction and sequencing for each prefecture (see Fig. 4, Table 3). Almost all fungal specimens were collected from adults of *S. esakii* and *S. taiwanensis* between April – June from 2004 to 2019.

Fig. 4. Southwestern part of Japan showing collection sites of *O. salganeicola* specimens used in this study. Specimens details are indicated in Table 3. The map is retrieved and edited from Geospatial Information Authority of Japan.

Taxonomy

Ophiocordyceps salganeicola Araújo, Moriguchi & Matsuura, sp. nov. – Mycobank MB836091; Fig. 5–6.

Etymology: Named after the host genus *Salganea*.

Specimen examined: Japan. Okinawa, Kunigami-son, Yonahadake, 26°43'45.0"N 128°12'48.2"E on *Salganea taiwanensis* (Blattodea, Blaberidae), 21 June 2017, M.G. Moriguchi, **Holotype:** XXX (to be provided by National Museum of Nature and Science - Japan).

External mycelium sparse, light to dark brown, arising from the host's sutures. One of two stromata, 1–7 cm long, 1.3–5 mm thick, cream to light or dark brown, clavate to cylindrical in shape. Perithecia immersed, usually covering the apical part descending until about the middle of the stromata, immersed to semi-immersed, ovoid to flask-shaped, (325–) 365 (–408) ' 100–140 µm. Asci hyaline, 8-spored, elongated clavate, 150–200 ' 7–11 µm with prominent cap. Ascospores hyaline, 70–100 x 3 µm, 7-septa, not disarticulating into partspores. *Hirsutella*-like phialides occurring sparsely on the surface of stromata where perithecia are absent, 7–16 ' 6–8 µm with neck measuring 18–30 ' 5–7.5. Conidia ovoid, 7 ' 5 µm, hyaline to pale brown.

Habitat. Forests of Miyazaki Prefecture, Yakushima and Okinawa islands of Japan. Host invariably dead inside rotten logs with only the fungus sprouting out.

Distribution. Only known for Japan.

Host association. *Salganea taiwanensis* and *S. esakii*.

Fig. 5. *Ophiocordyceps salganeicola* on *Salganea taiwanensis* (dried specimen) from Kunigami-son, Okinawa (Holotype – JPMA124). **a.** Two ascomata arising from *S. taiwanensis*. **b.** Close-up of dried ascoma; **c)** Cross section of ascoma showing the perithecial arrangement; **d.** Ascus with twisted ascospores; **e.** Perithecial ostiole; **f–g.** 8-celled ascospore.

Fig. 6. *Ophiocordyceps salganeicola* on *Salganea esakii* (fresh specimen) from Yakushima, Kagoshima (JPMA106). **a.** *Salganea esakii* with a single robust ascoma; **b.** Close-up showing early stage ascoma arising from ventral pronotum; **c–d.** Close-up of ascoma; **e.** Cross-section of ascoma; **f–g.** Perithecia; **h.** Ascospores within Ascus; **i–j.** 8-celled ascospores.

Molecular phylogeny and Evolutionary origins of cockroach-associated *Ophiocordyceps*

We obtained 20 new sequences from 5 specimens of *O. salganeicola* (Fig. 4, Table 3). Our phylogenetic analysis is in accordance with previously published *Ophiocordyceps* topology (Quandt et al. 2014, Sanjuán et al. 2015, Araújo et al. 2018, Tasanathai et al. 2019). All *O. salganeicola* specimens we collected, from different parts of Japan and infecting two species of *Salganea*, clustered together as a single species with considerably high degree of genetic similarity with a long branch (Fig. 7). It formed a monophyletic group with another cockroach-associated species, *O. blattae*, which is the type species for *Ophiocordyceps*. This is the first time *O. blattae* is included in a phylogenetic study.

Our results indicate that *Ophiocordyceps* originated from a beetle-associated ancestral (72% ACSR), corroborating with previous studies (Araújo & Hughes 2019). For the cockroach parasites, we found at least two independent origins within *Ophiocordyceps*, one within *Paraisaria* clade, i.e. *Paraisaria blattarioides* (Fig. 7 node A), and the other within the hirsutelloid species, i.e. *O. salganeicola* sp nov and *O. blattae* (Fig. 7 node B). The ancestral host association for the cockroach-associated *Paraisaria* lineage was ambiguously recovered, while for the hirsutelloid cockroach-associated species our data show it has originated likely from a termite-associated ancestor, although not strongly supported (44% ACSR). We also found that the association with termites is older than cockroaches, evolving independently at least twice (Fig. 7 nodes C and D). The oldest, would have arisen from beetles to termites (65% ACSR, Fig. 7 node C). However, the origins of *O. brunneirubra* remains uncertain as part of the ancient termite-associated lineage (Fig. 7 node C) or if it jumped more recently from Hymenoptera to termites (Fig. 7 Node C).

Paraisaria clade is an ecologically heterogeneous group composed by species parasitic on Coleoptera, Orthoptera, Lepidoptera and Hemiptera (Mongkolsamrit et al. 2019). Our ACSR analysis provided weak resolution for the origins of *O. amazonica*/*O. blattarioides*/*O. gracilis* clade with 50.1% for Orthoptera, 25.9% for Blattodea (cockroaches) and 10.9% for Lepidoptera (Fig. 7 Node A). Our data also did not provide strong support for the ancestral of *O. blattarioides* with 51.1% for Blattodea (cockroaches), 21% for Orthoptera and 20.6% for Lepidoptera (Fig. 7 Node A). Nevertheless, the whole *Paraisaria* lineage was strongly supported as evolved from a beetle parasite (Fig. 7 Node F, ACSR = 81.1%).

On the other hand, for the novel clade composed by *O. salganeicola* sp. nov. and *O. blattae*, our results suggest (BS=87; ACSR=72.4%) that it evolved from an ancestral parasite on termites (Fig. 7 – Node E). *Ophiocordyceps salganeicola*/*blattae* was retrieved as a sister group to a clade composed mostly by termite (Blattodea, Termitidae) parasites with also species associated with hemipterans (Pseudococcidae) and mites (Acari, Eriophyidae). According to our results, all host switches in this clade occurred from termites (i.e. termites to Coleoptera, termites to Hemiptera, termites to Acari and termites to

cockroaches). Unexpectedly, our analyses also suggest (ACSR=61.7%) the clade composed by parasites of Coleoptera, Lepidoptera and Hemiptera, including the economically and culturally important *O. sinensis*, could have been originated from an ancestor infecting termite, instead of beetle larvae as previously proposed (Araújo & Hughes 2019).

Fig. 7. Maximum likelihood tree of *Ophiocordycipitaceae* obtained from RAxML analyses based on a concatenated set of 5 genes (SSU, LSU, TEF, RPB1 and RPB2). Colored branches reflect Ancestral Character State Reconstruction (ACSR) analyses based on host associations (See legend at the bottom-left) and pie-charts represent the probability for the association with host orders. Host pictures by Alex Wild and Shizuma Yanagisawa.

Discussion

Ecology and Natural History of *Salganea*–*Ophiocordyceps* relationships

The insect body inherently protects itself from foreign microbes by its innate immunity, but at the same time offers a stable environment for the microbes that have already adapted to the host habitat and life cycles (Hurd 2003; Sadd & Schmid-Hempel 2008; Bright & Bulgheresi 2010, Araújo & Hughes 2016). This is particularly true for colonial cockroaches that spend most of their lives protected inside nests, for example some wood-feeding species within the families *Cryptocercidae* and *Blaberidae*, specifically the subfamily *Panesthiinae* (*Panesthiini*, *Ancaudeliini*, *Caepariini* and *Salganeini*). Among those groups, one of the most well-known social cockroaches is the genus *Salganea*, comprised by about 50 spp. (Beccaloni 2007, Bell et al. 2007, Wang et al. 2014). All the known species within the genus live within and feed on decaying wood, building chambers and galleries inside hardwood or coniferous logs that may take decades to degrade (Maekawa et al. 2008), providing long-term stable homeostatic conditions. Such protected environment certainly benefits indirectly the fungal parasites that are already inside the host body. An exposed cadaver on the forest floor would be much more susceptible to be eaten by scavengers or being consumed by other microorganisms, which would naturally antagonize with the fungal growth.

Salganea species form social groups, composed mostly by biparental families, consisting of a male-female and their offspring (Maekawa et al. 2008). Sociality endows insects with advantages such as increased efficiency of brood care, foraging and anti-predator defenses. However, infectious diseases can potentially spread more easily within a colony because of their high densities, frequent social contact and also that group members are often close relatives and thus susceptible to the same parasitic infections (Cremer et al. 2007). Therefore, it is surprising that only three species of *Ophiocordyceps*, a common and widespread group of entomopathogenic fungi, were recorded infecting the equally diverse and globally distributed cockroaches (Bourguignon et al. 2018). *Ophiocordyceps* species infecting social insects, notably ants, are one of the most broadly distributed and ubiquitous entomopathogenic fungi in tropical forests worldwide (Araújo et al. 2015, 2018). They often form epizootic events, in which hundreds of infected ants can be found a small patch of forest (Evans and Samson 1982, Pontoppidan et al. 2009).

On the other hand, however, *Ophiocordyceps* on social cockroaches are rare in Japan and only one or two infected individuals are collected in the same log, despite the ubiquity and abundance of hosts in one area.

Based on our extensive field surveys we found that fruiting bodies of *O. salganeicola* in Okinawa start to emerge in decaying logs in early April. However, they seem to require at least a few months to be mature and develop the sexual morph in the field. This development occurs in parallel with the mating season of the host cockroaches from April to July when newly emerged adults leave their logs and parents, fly, mate and burrow into a new nest (Osaki Haruka, personal communication). Presumably, these young adults might become infected by the ascospores/conidia of *O. salganeicola* during colonization of a new log. The host is then later killed and consumed by the fungal parasite, eventually producing new fruiting bodies in the next mating season. There has been no record of an infected nymph out of more than 26 fungal specimens observed in Okinawa, but only a single infected nymph (see Fig. 2-E) out of more than 27 specimens in Yakushima between 2015–2019, suggesting the primary targets of this fungus are newly emerged adults. However, this proposed lifecycle of *O. salganeicola* is only hypothetical and requires periodical field observations in the same ecological habitat along with host insect behaviors (Maekawa et al. 2008).

Behavior manipulation

The behavior manipulation caused by *Ophiocordyceps* fungi on their hosts is a striking phenomenon, especially on species associated with ants, the so-called “zombie-ant fungi” (Andersen et al. 2009, Araújo et al. 2018). It has been posited that species within the *Ophiocordyceps unilateralis* core clade infecting Camponotini ants evolved such an ability as a response to the strong social immunity displayed by ant societies that prevents fungal transmission and development inside the colony (Araújo & Hughes 2019). Conversely, as far as we know, there is no evidence of social cockroaches recognizing the infected members of their colony, except for the parental and sibling’s grooming behavior that might fend off superficial parasites. Thus, fungal infection, development and transmission can potentially occur in the same log where other members of the colony still inhabit, implying no drastic behavior manipulation is needed in order to remove the host from its nest and thus complete the parasite’s life-cycle. However, there is a possibility of a subtle manipulation.

Salganea cockroaches inhabit deep inside the trunk, just becoming exposed to the external environment in the mating season, whereas the *Ophiocordyceps*-infected ones are found only few centimeters below the wood surface (Fig. 2). Thus, we could speculate that the fungus might potentially be able to manipulate host’s behavior by leading to a migration towards a more superficial layer inside the log. This host migration could be stimulated by the need of a more oxygen- and water-rich stratum and/or attraction to the light coming from an opening, through which the fungus sprout outwards to further shoot its spores (Fig. 2, A–B). Future studies could test this hypothesis and help us to clarify such a speculation.

Host Association, Speciation and Distribution

While some distinct – mostly macro – morphological features can be observed on *O. salganeicola* infecting both host species that diverged from a single ancestor only around 4–5 mya (Maekawa et al. 1999, Maekawa & Matsumoto 2003), nucleotide sequences are highly conserved among diverse strains in wide range of geographic regions and islands. The number of polymorphic sites within all five gene sequences from multiple samples was only 2 out of 4,202 and both were synonymous. Thus, geographic and reproductive isolation of the fungal strains may have not yet resulted in the allopatric speciation of *O. salganeicola*. The long branch length and high host specificity to the genus *Salganea* indicate a long co-evolutionary relationship with the host populations and unlikelihood of recent host jumping (Fig. 7, Node B). However, we still do not know whether the single parasite strain can only persist in one geographic region and/or island infecting the same host populations over generations, or jump across multiple close-related host populations and species horizontally even after such a long geographic isolation of the Ryukyu islands. In some of these islands such as Amamioshima and Tokunoshima in Kagoshima and Ishigaki-jima and Iriomote-jima in Okinawa, there has been no collection record of *O. salganeicola*, suggesting the loss of entomoparasite strains in these host populations. These questions on the evolution of host specificity and associations of *O. salganeicola* in the Japanese archipelago of Ryukyus deserve particular attention for studying host-parasite co-evolution in the context of island biology, which can be possibly be tested by the field-collected fungal ascoma and laboratory-reared *Salganea* colonies from different islands in future studies.

Conclusion

The Japanese fungal community, remarkably the entomopathogens, likely harbors one of the largest reservoirs of undocumented fungal species in the world. We still know very little about these organisms and their ecological roles in the environment and dynamic associations with host insects, including blattodean-associated ones such as *O. salganeicola*. Therefore, biodiversity studies are crucial to understand their true diversity with the invaluable help by amateur and professional mycologists. As we move forward and describe more and more species through microscopic and molecular tools, new insights into the evolutionary origins of these organisms are revealed, as well as their ecological associations with the insect hosts. Currently, there is still a substantial gap on the knowledge about insect ecology and fungal biology along the context of host-parasite interactions and their life cycles. In this study, our goal was to describe an ecologically rare fungal species parasitizing unique social insects, and to provide some insights into their evolution by considering natural histories of both the parasite and its host. Thereby, we expect that this study will contribute to the understanding of one of the most prolific and diverse groups of entomopathogenic fungi, the genus *Ophiocordyceps*.

Declarations

Acknowledgments

We thank Hiroaki Yamashita for sample collection in Yakushima, Shizuma Yanagisawa for *Salganea* cockroach photos, Haruka Osaki for the information on the host insect behaviors, Gaku Tokuda, Kiyoto

Maekawa, and Christine Nalepa for the advice on the insect taxonomy and ecology, Pepijn Kooij and Lee Davis from Kew Gardens for the efforts to get DNA data from the holotype of *O. blattae* and Jennifer Luangsa-ard and Wasana Noisripoom for providing DNA sequences of *O. blattae* found in Thailand. The authors also would like to highlight the extreme importance of work done previously, especially by Daisuke Shimizu and Yosio Kobayashi, who have inspired several generations of professional and amateur mycologists with their art and science. J.P.M.A. conducted research as an invited foreign researcher at the University of the Ryukyus. This study was supported by JSPS Grant-in-Aid KAKENHI Grant No. 18K19217, 18KK0211 and by a general research grant of the Institute of Fermentation, Osaka (IFO) to Y.M.

References

1. Andersen SB, Gerritsma S, Yusah KM, Mayntz D, Hywel-Jones NL, Billen J, Boomsma JJ, Hughes DP (2009) The life of a dead ant: the expression of an adaptive extended phenotype. *Am Nat* 174:424–433
2. Araújo JPM, Evans HC, Geiser DM, Mackay WP, Hughes DP (2015) Unravelling the diversity behind the *Ophiocordyceps unilateralis* (Ophiocordycipitaceae) complex: three new species of zombie-ant fungi from the Brazilian Amazon. *Phytotaxa* 220:224–238
3. Araújo JPM, Evans HC, Kepler R, Hughes DP (2018) Zombie- ant fungi across continents: 15 new species and new combinations within *Ophiocordyceps*. I. Myrmecophilous hirsutelloid species. *Stud Mycol* 90:119–160
4. Araújo JPM, Hughes DP (2016) Diversity of entomopathogenic fungi: which groups conquered the insect body? *Adv Genet* 94:1–39
5. Araújo JPM, Hughes DP (2019) Zombie-ant fungi emerged from non-manipulating, beetle-infecting ancestors. *Curr Biol* 29:3735–3738
6. Beccaloni, GW (2007) Blattodea Species File Online. Version 5.0/5.0. World Wide Web electronic publication. <<http://Blattodea.SpeciesFile.org>> [accessed 8 July 2020] (A regularly updated world catalogue of extant Blattodea excluding the Termitoidae (termites))
7. Bell WJ, Roth LM, Nalepa CA (2007) *Cockroaches: Ecology, Behavior, and Natural History*. The Johns Hopkins University Press, USA
8. Blackwell M, Gilbertson RL (1981) *Cordycepioideus octosporus*, a Termite Suspected Pathogen from Jalisco. Mexico *Mycologia* 73:358–362
9. Bourguignon T, Tang Q, Ho SYW, Juna F, Wang Z, Arab DA, Cameron SL, Walker J, Rentz D, Evans TA, Lo N (2018) Transoceanic Dispersal and Plate Tectonics Shaped Global Cockroach Distributions: Evidence from Mitochondrial Phylogenomics. *Mol Biol Evol* 35:970–983
10. Bright M, Bulgheresi S (2010) A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* 8:218–230

11. Castlebury LA, Rossman AY, Sung G-H, Hyten A, Spatafora JW (2004) Multigene phylogeny reveals new lineage for *Stachybotrys chartarum*, the indoor air fungus. *Mycol Res* 108:864–872
12. Crawley H (1905) *Coelosporidium blattellae*, a new sporozoan parasite of *Blattella germanica*. *Academy of Natural Sciences of Philadelphia* 57:158–161
13. Cremer S, Armitage SAO, Schmid-Hempel P (2007) Social immunity. *Current Biology* 17.: 693–702
14. Evangelista DA, Wipfler B, Béthoux O, Donath A, Fujita M, Kohli MK, Legendre F, Liu S, Machida R, Misof B, Peters RS, Podsiadlowski L, Rust J, Schuette K, Tollenaar W, Ware JL, Wappler T, Zhou X, Meusemann K, Simon S (2019) An integrative phylogenomic approach illuminates the evolutionary history of cockroaches and termites (Blattodea). *Proceedings of the Royal Society B* 286: 1–9
16. Evans HC, Samson RA (1982) Cordyceps species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems. I. The *Cephalotes* (Myrmicinae) complex. *Transactions of the British Mycological Society* 79:431–453
17. Gomez-Polo P, Ballinger MJ, Lalzar M, Malik A, Ben-Dov Y, Mozes-Daube N, Perlman SJ, Iasur-Kruh L, Chiel E (2017) An exceptional family: Ophiocordyceps-allied fungus dominates the microbiome of soft scale insects (Hemiptera: Sternorrhyncha: Coccidae). *Mol Ecol* 26:5855–5868
18. Hurd W (2003) Manipulation of Medically Important Insect Vectors by Their Parasites. *Annual Review of Entomology* 48:141–161
19. Katoh K, Standley KDM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30:772–780
20. Luangsa-ard JJ, Tasanathai K, Thanakitpipattana D, Khonsanit A, Stadler M (2018) Novel and interesting *Ophiocordyceps* spp. (Ophiocordycipitaceae, Hypocreales) with superficial perithecia from Thailand. *Stud Mycol* 89:125–142
21. Maekawa K, Lo N, Kitade O, Miura T, Matsumoto T (1999) Molecular Phylogeny and Geographic Distribution of Wood-Feeding Cockroaches in East Asian Islands. *Molecular Phylogenetic Evolution* 13:360–376
22. Maekawa K, Matsumoto T (2003) Biogeography of Japanese wood-feeding cockroaches genus *Salganea* and *Panesthia* (Blaberidae: Panesthiinae). *Molecular Phylogenetics Evolution* 27:156–159
23. Maekawa K, Matsumoto T, Nalepa CA (2008) Social biology of the wood-feeding cockroach genus *Salganea* (Dictyoptera, Blaberidae, Panesthiinae): did ovoviviparity prevent the evolution of eusociality in the lineage? *Insectes Soc* 55:107–114
24. Matsuura Y, Moriyama M, Łukasik P, Vanderpool D, Tanahashi M, Meng XY, McCutcheon JP, Fukatsu T (2018) Recurrent symbiont recruitment from fungal parasites in cicadas. *Proceedings of National Academy of Sciences U.S.A.* 115: 5970–E5979
25. Mongkolsamrit S, Noisriboom W, Arnarnart N, Lamlertthon S, Himaman W, Jangsantear P, Samson RA, Luangsa-ard JJ (2019) Resurrection of *Paraisaria* in the Ophiocordycipitaceae with three new species from Thailand. *Mycological Progress* 18:1213–1230

26. Petch T (1924) Studies in entomogenous fungus. IV Some Ceylon Cordyceps Transactions of the British Mycological Society 10:28–45
27. Petch T (1931) Notes on entomogenous fungi. Transactions of the British Mycological Society 16:55–75
28. Petch T (1934) Notes on entomogenous fungi. Transactions of the British Mycological Society 19:161–194
29. Pontoppidan M-B, Himaman W, Hywel-Jones NL, Boomsma JJ, Hughes DP (2009) Graveyards on the move: the spatio-temporal distribution of dead *Ophiocordyceps*-infected ants. PLoS One 4(3):e4835
30. Purrini K, Weiser J, Kohring GW (1988) *Coelosporidium tangae* n. sp. (Protista), a new protist parasitizing a natural population of a field cockroach, *Blatta* sp. (Blattaria). Arch Protistenk 136:273–281
31. Quandt CA, Kepler RM, Gams W, Araújo JPM, Ban S, Evans HC, Hughes D, Humber R, Hywel-Jones N, Li Z, Luangsa-ard JJ, Rehner SA, Sanjuán T, Sato H, Shrestha B, Sung G-H, Yao Y-J, Zare R, Spatafora JW (2014) Phylogenetic-based nomenclatural proposals for Ophiocordycipitaceae (Hypocreales) with new combinations in *Tolypocladium*. IMA Fungus 5:121–134
32. Sadd BM, Schmid-Hempel P (2009) Perspective: Principles of ecological immunology. Evol Appl 2:113–121
33. Sanjuan TI, Fanco-Molano AE, Kepler RM, Spatafora JW, Tabima J, Vasco-Palacios AM, Restrepo S (2015) Five new species of entomopathogenic fungi from the Amazon and evolution of neotropical *Ophiocordyceps*. Fungal Biology 119:901–916
34. Schall C, Gautier JY, Bell WJ (1984) Behavioural Ecology of Cockroaches. Biol Rev 59:209–254
35. Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313
36. Stiffler CB (1941) A New Genus of Hypocreales. Mycologia 33:82–86
37. 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
38. Tسانathai K, Noisripoom W, Chaitika T, Khonsanit A, Hasin S, Luangsa-ard JJ (2019) Phylogenetic and morphological classification of *Ophiocordyceps* species on termites from Thailand. MycoKeys 56:101–129
39. Vilgalys R, Sun BL (1994) Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. Proceedings of National Academy of Sciences 91: 4599–4603
40. Wang X, Shi Y, Wang Z, Che Y (2014) Revision of the genus *Salganea* Stål (Blattodea, Blaberidae, Panesthiinae) from China, with descriptions of three new species. ZooKeys 412:59–87
41. Woolever P (1966) Life history and electron microscopy of a haplosporidian, Nephridiophaga blattellae (Crawley) n. comb., in the Malpighian tubules of the German cockroach, *Blattella germanica* (L.). Journal of Protozoology 13:622–642

Figures

STUDIES IN ENTOMOGENOUS FUNGI.

(With Plate I and 3 Text-figs.)

IV. SOME CEYLON CORDYCEPS.

By *T. Petch, B.A., B.Sc.*

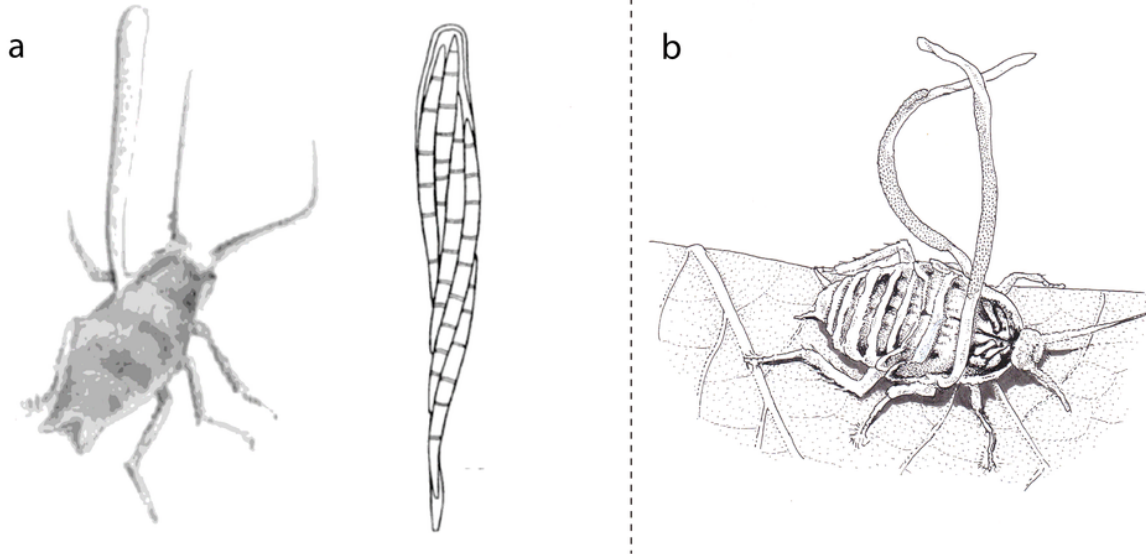


Figure 1

Ophiocordyceps blattae illustrations. a. the holotype original illustration of the host with fungus arising laterally and ascus with septate ascospores (adapted from Petch 1924); b. Illustration of *O. blattae* from Thailand used in this study (the actual specimen), with two ascomata arising laterally on both sides.



Figure 2

Ophiocordyceps salganeicola emerging from *Salganea esakii* in its natural habitat in Yakushima, isl. Kagoshima. A-B. Visible part of the ascoma, erupting from a hole in the wood; C-D. Hosts buried in the wood, visible only after digging few centimeters; E. Infected host on its nymphal stage, very rare.

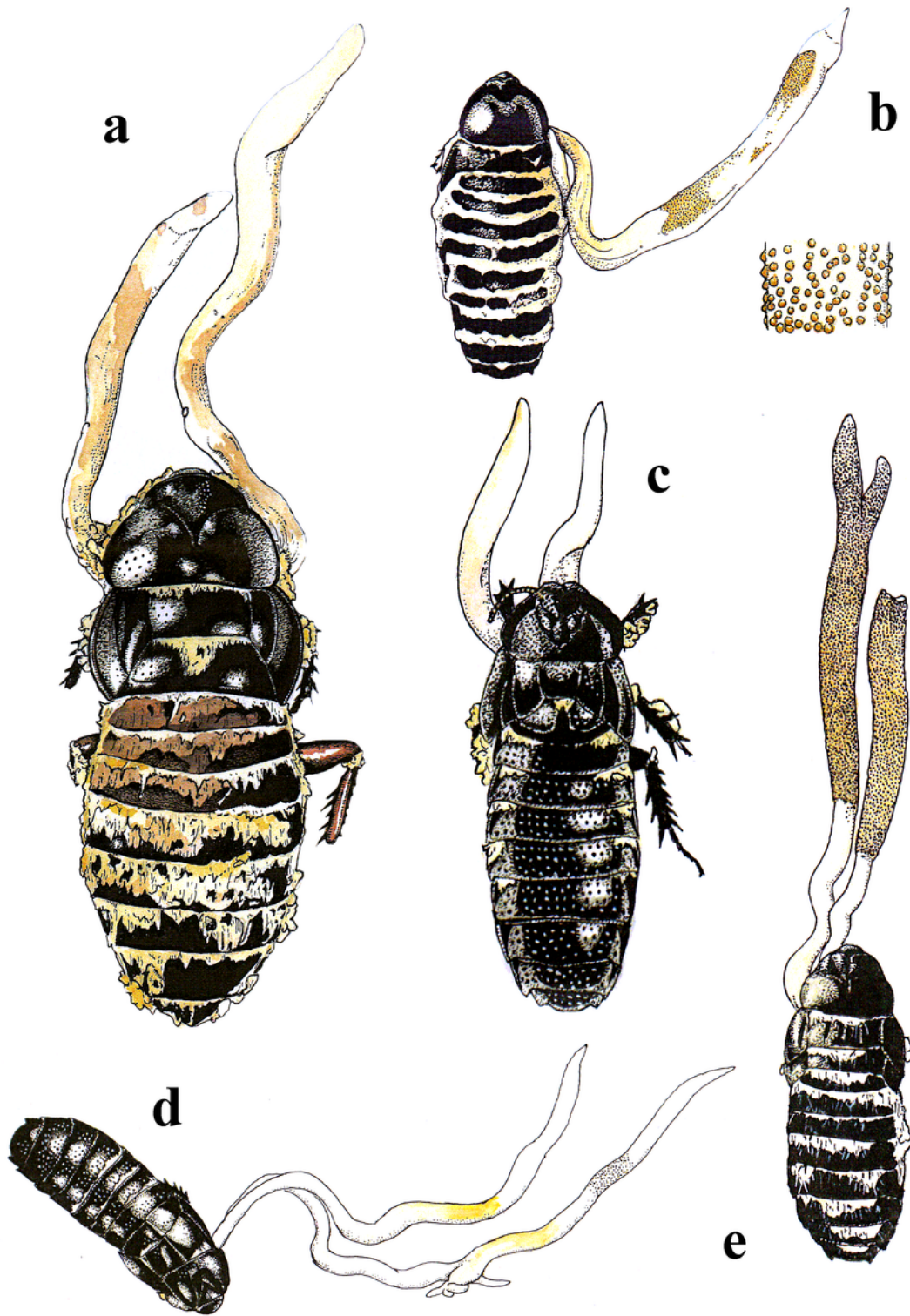


Figure 3

Illustrations of *Ophiocordyceps salganeicola* at various stages of development. A. an illustration of a teleomorph of *O. salganeicola* growing on *Salganea esakii* collected in Miyazaki on June 22, 2005. B. teleomorph roach on *S. esakii* collected in Yakushima on June 17, 2017 and a closeup illustration of perithecia. C-E. distinct developmental stages of *O. salganeicola* on *S. taiwanensis*, collected in Katsudake, Nago-shi, Okinawa Apr 23, 2016 D. Mori04 and E. Kunigami-son, Okinawa on May 29, 2016.

ii

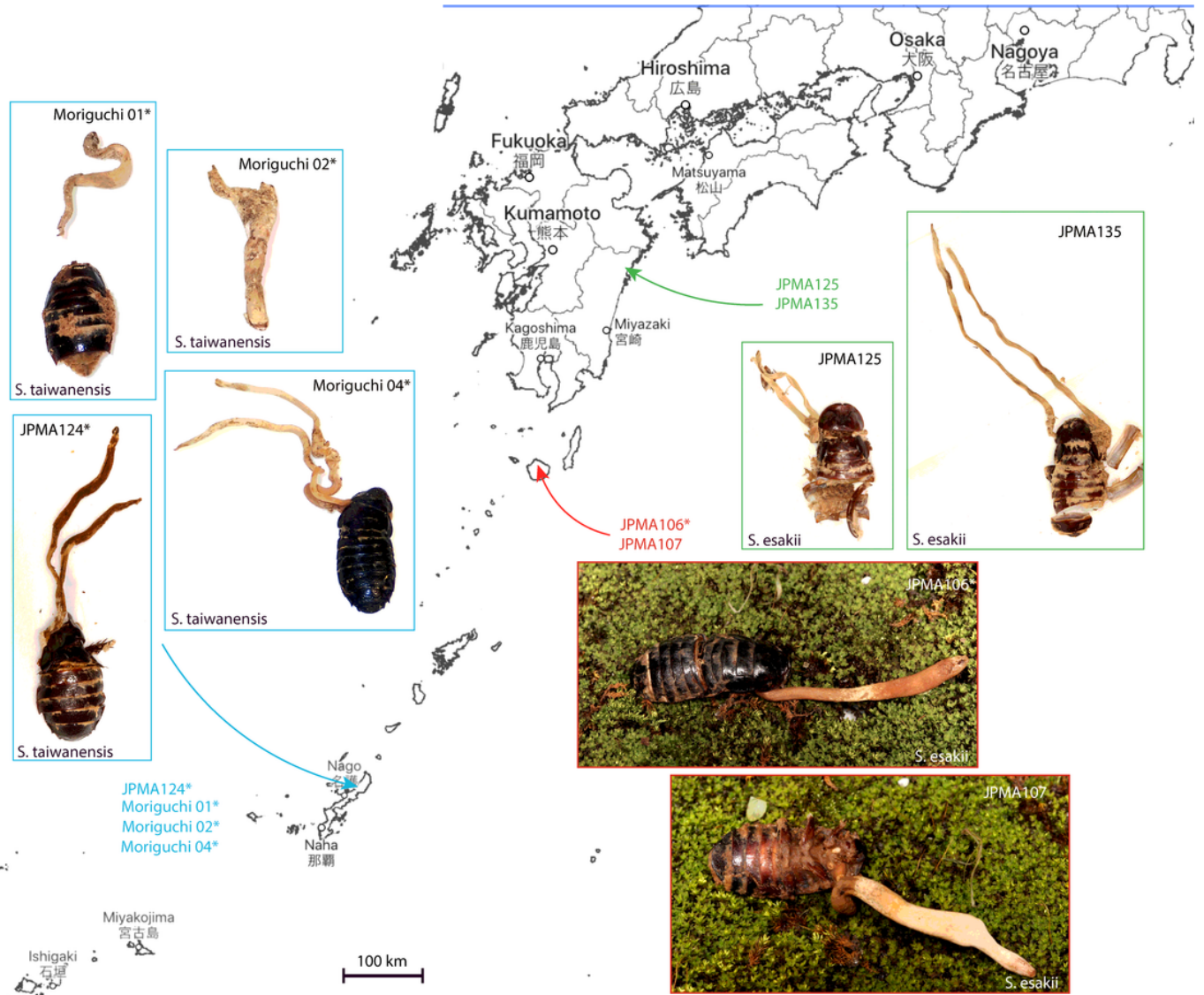


Figure 4

Southwestern part of Japan showing collection sites of *O. salganeicola* specimens used in this study. Specimens details are indicated in Table 3. The map is retrieved and edited from Geospatial Information Authority of Japan.

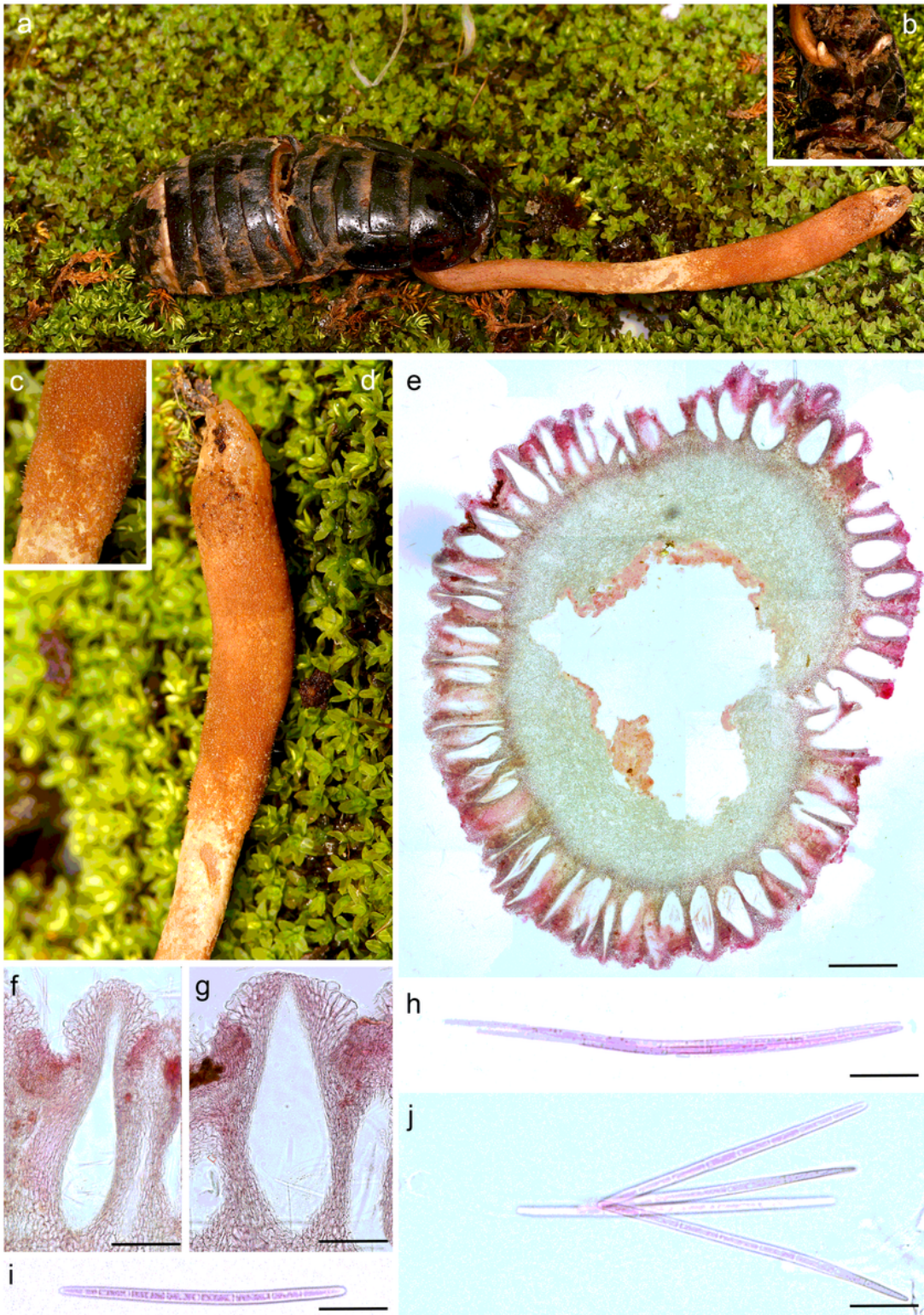


Figure 5

Ophiocordyceps salganeicola on *Salganea taiwanensis* (dried specimen) from Kunigami-son, Okinawa (Holotype – JPMA124). a. Two ascomata arising from *S. taiwanensis*. b. Close-up of dried ascoma; c) Cross section of ascoma showing the perithecial arrangement; d. Ascus with twisted ascospores; e. Perithecial ostiole; f–g. 8-celled ascospore.



Figure 6

Ophiocordyceps salganeicola on *Salganea esakii* (fresh specimen) from Yakushima, Kagoshima (JPMA106). a. *Salganea esakii* with a single robust ascoma; b. Close-up showing early stage ascoma arising from ventral pronotum; c–d. Close-up of ascoma; e. Cross-section of ascoma; f–g. Perithecia; h. Ascospores within Ascus; i–j. 8-celled ascospores.

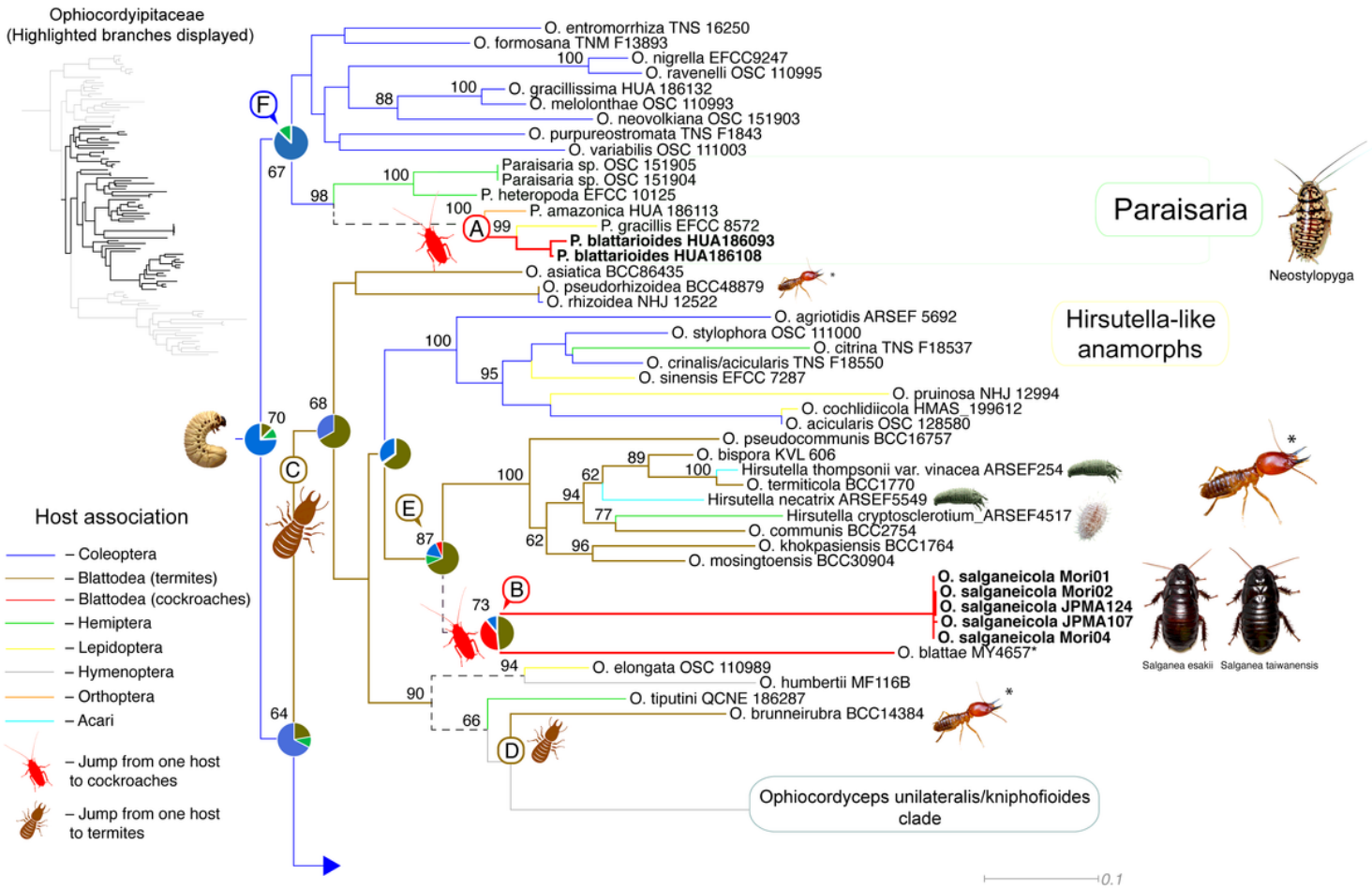


Figure 7

Maximum likelihood tree of Ophiocordycipitaceae obtained from RAxML analyses based on a concatenated set of 5 genes (SSU, LSU, TEF, RPB1 and RPB2). Colored branches reflect Ancestral Character State Reconstruction (ACSR) analyses based on host associations (See legend at the bottom-left) and pie-charts represent the probability for the association with host orders. Host pictures by Alex Wild and Shizuma Yanagisawa.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1Species.xlsx](#)
- [Table2GenBank.xlsx](#)
- [Table3specimens.xlsx](#)