The entomophagous caterpillar fungus *Ophiocordyceps sinensis* is consumed by its lepidopteran host as a plant endophyte

Zhengyang Wang a,*, Meng Li b, c, Wenbin Ju b, Wenqing Ye d, Longhai Xue e, David E. Boufford f, Xinfen Gao c, Bisong Yue g, Yong Liu h, Naomi E. Pierce a, **

a Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, USA
b Chengdu Institute of Biology, Chinese Academy of Sciences, China
c Co-Innovation Center for Sustainable Forestry in Southern China, College of Biology and the Environment, Nanjing Forestry University, China
d Systematic & Evolutionary Botany and Biodiversity Group, MOE Laboratory of Biosystem Homeostasis and Protection, College of Life Sciences, Zhejiang University, China
e Institute of Plant Protection, Sichuan Academy of Agricultural Sciences, China
f Harvard University Herbaria, Harvard University, USA
g The College of Life Sciences, Sichuan University, China

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1. Introduction

Endophytic insect pathogenic fungi (EIPF) are insect-infesting fungi that also have the ability to establish themselves within plant tissues (Raman et al., 2012; Barelli et al., 2016; Moonjely et al., 2016). Ecologically, EIPF are often considered to form an alliance with their host plants because they can defend the plants against insect herbivores. As such, EIPF are good candidates to develop as commercial insecticides (Sánchez-Rodríguez et al., 2018). Several EIPF species enhance growth when host plants have been experimentally inoculated (Sasan and Bidochka, 2012; Liao et al., 2014). More specifically, *Metarhizium robertsii*, the model organism of EIPF, is able to translocate insect-derived nitrogen to plants and receive carbon from plants in return (Behie et al., 2012, 2017).

Although an insect-pathogenic lifestyle has convergently evolved multiple times in fungi, the ability to double task as both plant endophyte and insect pathogen has only been observed in the fungal order Hypocreales (phylum: Ascomycota, class: Sordariomycetes) (see Braine et al., 2019 for review). EIPF use homologous genes in the penetration of both plant cell walls and insect
exoskeletons (Wang and St Leger, 2007; Zhang et al., 2019). The ancestral state of some grass-associated EIPF in the family Clavicipitaceae (order: Hypocreales) was animal pathogenic (Spatatofora et al., 2007), but recent phylogenomic analyses suggest the common ancestor of the order Hypocreales is plant pathogenic, experiencing cross-kingdom host shifts from plants to insects, and reversion back to plant endophytes in some lineages (Wang et al., 2016; Zhang et al., 2018). Signals of introgression of genes related to host recognition have also been identified among many hypocrelean fungi (Zhang et al., 2018). These data suggest that hypocrelean fungi currently described as solely entomopathogenic might also be capable of a plant-endophytic lifestyle.

Ophiocordycipitaceae is often described as an entomopathogenic fungus that parasitizes lepidopteran larvae of the ghost moth genus Thitarodes (family: Hepialidae) (Sung et al., 2007; Shrestha et al., 2014). Ophiocordycipitaceae infection of Thitarodes larvae produces a sclerotium whose base is enclosed within the larval exoskeleton. This mummified caterpillar sclerotium, given the name “caterpillar fungus,” was documented as early as the 16th century in Tibetan medicinal texts and is still coveted in eastern Asia as a traditional herbal medicine (Winkler, 2008, 2010). The fungus is only found on high elevation meadows of the eastern Himalayas, Qinghai-Tibetan Plateau and the Hengduan Mountains (e.g. Wang and Yao, 2011; Wang et al., 2019b) and its declines in harvest have been attributed to climate change and over-collection (e.g. Yan et al., 2017; Hopping and Ree, 2017). Zhong et al. (2014) identified the presence of Ophiocordycipitaceae, and its different lineages have been described both as plant endophytes and insect parasites (Gazis et al., 2014). Second, in its natural habitat, O. sinensis infects Thitarodes larvae. Although described as an insect-fungus symbiosis, involvement of a plant-endophytic stage in O. sinensis infection of insect larvae has been hypothesized for two reasons. First, many taxa in Hypocreales, including model EIPF genera such as Metarhizium and Beauveria, have plant-endophytic lifestyles when not conspicuously infecting other hosts (see review by Kepler et al., 2017). For example, the genus Polypodcladium belongs to the same family as O. sinensis (Ophiocordycipitaceae), and its different lineages have been described both as plant endophytes and insect parasites (Gazis et al., 2014). Second, in its natural habitat, O. sinensis disperses through airborne ascospores above ground, and its concentration in the soil (outside the vicinity of a sporulating stroma) is low, while all known Thitarodes larval hosts are soil-dwelling (see Zhou et al., 2014; Tao et al., 2015 for descriptions of Thitarodes life history). The low concentration of O. sinensis in the soil suggest that it may be difficult for the fungus to infect its soil-dwelling insect host (Peng et al., 2013). Efforts to artificially inoculate Thitarodes larvae and commercially cultivate caterpillar fungi have been unsuccessful at a large scale without plant mediation (Zhou et al., 2014).

Alternatively, it has been hypothesized that dispersed O. sinensis ascospores landing on external plant tissues can penetrate and subsequently reside in plants as endophytes, which in turn infect soil-dwelling Thitarodes larvae when they consume plant root tissues (Lei et al., 2015). The implication of this hypothesis is that direct consumption of endophytic O. sinensis in plant roots is an important pathway of host infection. In support of this hypothesis, Zhong et al. (2014) showed the presence of O. sinensis in roots of 23 species of plants in a caterpillar fungus habitat. Lei et al. (2015) detected O. sinensis in both leaf and root tissue of 6 plant species and experimentally demonstrated that plant extracts promote the growth of O. sinensis mycelia in culture. Lei et al. (2011) used multiple target PCR primers to establish that at a single caterpillar fungus collection site in Tibet, Thitarodes larvae fed on the tender roots of alpine plants belonging to 24 genera in 16 families. At the time of the larval diet study conducted by Lei et al. (2011), investigating the gut content of polyphagous insects involved diagnostic PCR with multiple primers, each designed to detect a single known plant species. This required prior knowledge of a generalist’s diet and tailored primer design to distinguish each taxon present in the diet (Staudacher et al., 2011; Garcia-Robledo et al., 2013; Brim et al., 2018; Wang et al., 2019a; Zhu et al., 2019). Modern methods now allow high-throughput next generation sequencing (NGS) for pools of amplicons generated for plant barcode sequences from insect gut contents, making it possible to quantify each diet content as a characteristic subsample of the plant community. For example, based on differences in diet contents, researchers have been able to construct food networks of leaf beetles (Kajtoch et al., 2015) and verify the movement patterns of psyllid bugs (Cooper et al., 2019). While the accuracy of NGS-based insect diet detection is restricted by sequencing depth and can show individual inconsistency, the method is nevertheless well-suited for investigating a generalist insect diet when the range of consumable plants is known (Rennstam Rubbmark et al., 2019).

Apart from verifying the hypothesized O. sinensis infection pathway from plant root to caterpillar gut, understanding the diet of Thitarodes larvae at an individual level will help formulate questions regarding the role of plants in dispersing EIPF from a community ecology perspective. The geographical region where O. sinensis is distributed is a biodiversity hotspot that contains 12,000 species of vascular plants, many of which diversified in high elevation meadows and valleys (Rouford and van Dijk, 1999; Xing and Ree, 2017), Zhong et al. (2014) identified more than 42 species of plants that could be potentially consumed by larvae in a single caterpillar fungus habitat. Considering the wide range of plant species available for consumption to any Thitarodes larva, NGS-based diet analysis at the resolution of individual gut content can help us investigate whether O. sinensis preferentially partners with certain plants as EIPF and whether Thitarodes larvae preferentially consume certain plants. In addition, if individual diets differ in composition, considering diets as communities will allow us to ask whether the composition and diversity of an individual’s diet predicts its level of fungal infection.

In this study, we combine field screening of endophytic O. sinensis and NGS sequencing of larval diet to investigate whether plants with endophytic O. sinensis are consumed by host larvae. We screened all plants for O. sinensis endophytic presence in both roots and leaves at a single caterpillar fungus collection site at the eastern side of Mt. Gongga, Sichuan, China. We hypothesize that if the ascospore-dispersed O. sinensis consists of a plant mediated endophytic life cycle (which transfers O. sinensis from leaf to root), we would see high association of endophytic O. sinensis presence in plant leaf correlated with endophytic O. sinensis presence in plant roots. We also analyzed whether O. sinensis preferentially partners with certain lineages of plants in the habitat. We then analyzed the diet of Thitarodes larvae living in the soil in the same habitat to test the hypothesis that plant species containing O. sinensis in their roots are consumed by the larvae. Finally, we analyzed whether larval diet-based parameters such as breadth and specificity predict the level of fungal infection in individual Thitarodes larvae.

2. Materials and methods

2.1. Endophyte detection

Plants were sampled at the Haizidang caterpillar fungus collection site in an area of around 0.48 km² in Yanzigou valley, Mt.
At Haizidang, *T. shambalaensis* larvae were sampled by randomly excavating 1 m² plots 30 cm deep across the collection site. The inverted vegetation was restored after sampling to minimize environmental disturbance. Collected larvae were visually inspected and scored for size as follows: level 1 (≤1 cm); level 2 (1–2 cm); level 3 (2–2.5 cm); level 4 (>2.5 cm). The extent of abnormal body coloration and activity as proxy for *O. sinensis* infection were scored as follows: level 1 (head light yellow); level 2 (upper body yellow); level 3 (severe infection; body is completely yellow, difficulty in movement). Collected larvae were surface-sterilized; the midgut of the contents of each larval gut were extracted with a Tiangen Genomic DNA kit. A fragment of the large subunit of the ribulose bisphosphate carboxylase (rbcL) gene region was then amplified (Z1aF and hp2R primers, ~175 bp, see Hofreiter et al., 2000) and sequenced on Illumina MiSeq (pair-end 250 bp). Sequences were processed using the QIIME pipeline (version 1.8.0, Caporaso et al., 2010). They were filtered by sequencing quality and length (>150 bp), de-replicated and checked for chimera sequences using USEARCH quality filter (Edgar, 2010). Post-filtering chimera-free sequences were assigned to operational taxonomic units (OTUs) at 97% sequence identity using UCLUST (Edgar, 2010), and OTUs consisting of less than 0.001% of the sequences were removed. Family level taxonomic identity of each OTU was assigned using NCBI database (NCBI Resource Coordinators, 2016) as reference.
with root *O. sinensis* detection in all plant families in Haizidang, (5) the phylogenetic correlation of its diet with vegetation type and (6) the phylogenetic correlation of its diet with plant local abundance.

We visualized the plant diversity within the gut contents of all individuals using both principal component analysis (PCA, plotting the loadings of each plant family on the first two PCs) and principal coordinates analysis (PCoA, from the unweighted UniFrac distance matrix) (Lozupone and Knight, 2005). We performed PERMANOVA tests for categorical variables and MANTEL tests for continuous variables in the R package "vegan" (Oksanen et al., 2013) to examine whether there were significant differences in dietary composition between different groups of larvae: infection levels and larval size were analyzed both as continuous variables (numeric levels noted in the field) and categorical variables (“bigger” or “smaller” than 2 cm; presence/absence of level 3 severe infection of *O. sinensis*). Unweighted UniFrac distances were used to calculate the distance matrices needed for these tests to account for phylogenetic signal.

### 3. Results

At Haizidang, a total of 115 sample plant species were collected and identified to 57 genera in 27 families (including 2 unidentifiable samples in the phylum Bryophyta, Table S1). Plant endophytic *O. sinensis* was detected in 42 samples representing 52.6% of the genera and 66.6% of the families, with 31 detections in leaf tissues and 25 detections in root tissues (Fig. 1, S1, Tables S1 and S2). Presence of *O. sinensis* in a plant sample’s roots was significantly correlated with its presence in the same plant sample’s leaves, but not with the vegetation type, or the local abundance of the plant (Table 1). None of the plant characteristics measured (presence of endophytic *O. sinensis*, vegetation type, local abundance) showed significant phylogenetic signal (all D > 0). At the genus and family level, the presence/absence of endophytic *O. sinensis* showed a significant signal of over-dispersion across the phylogeny (D > 1) (Table 2).

Gut contents of 73 *T. shambalaensis* larvae (n = 8 in 2017, n = 65 in 2018) were sequenced with rbcL primers, resulting in 41,204 reads per sample (s.d. = 13,596). Each sample contained on average 116 OTUs (s.d. = 47.8). Using families detected in Haizidang as a filter, we retained 46.5% (s.d. = 0.31) of the OTUs in each sample, representing 22 of the 27 families collected in the habitat (Fig. 2, S2). The four OTU families that were most abundant by OTU counts were: Polygonaceae (21.6%), phylum Bryophyta (17.3%), Ranunculaceae (9.7%), and Poaceae (8.3%). OTUs in families Cyperaceae, Rosaceae, Papaveraceae, Fabaceae and Brassicaceae each contributed 1–5% of the total OTUs. Other plant families were represented by less than 1% of OTUs. The most commonly present plant families in the sample guts were: phylum Bryophyta (64 samples), Polygonaceae (60 samples), Papaveraceae (34 samples), Ranunculaceae (34 samples), Fabaceae (31 samples), Poaceae (29 samples), Rosaceae (26 samples) and Asteraceae (23 samples) (Table S3). Of the 22 plant families consumed by larvae, 12 (54.6%) had endophytic *O. sinensis* in their root tissue. Plant families consumed by the larvae as a whole were not phylogenetically clustered (D = 0.16, p = 0.33), and plant family presence in the gut was also not correlated with any of the field measured traits, including local abundance, vegetation type, or the presence of *O. sinensis* in the roots. Individual larvae were recorded to feed on anywhere between 1 and 12 families of plants (mean = 5.43, s.d. = 2.73). Phylogenetic signals of individual diets ranged from highly conserved to highly over-dispersed (DEmax = 2.59, DEmin = −3.75, mean = −0.3847, s.d. = 1.23). Most individuals showed no dietary preference for any plant trait measured in this study, except for 2 larvae whose plant preferences were strongly associated with the presence of endophytic *O. sinensis* in the root (Fig. 3), and 5 larvae that showed significant preferences for vegetation type (Fig. 53).

Among the 55 larvae that we assessed for *O. sinensis* infection, we identified 11 as being severely infected (score level 3: completely dark yellow exoskeleton and severe difficulty in movement). All eight larvae collected in 2017 were severely infected (Table S4); while only three samples in 2018 were severely infected. We, therefore, did not include the 2017 data in our regression analyses because they are confounded with infection level. Larval size, not composition and diversity of an individual's

### Table 1

<table>
<thead>
<tr>
<th>Presence of <em>Ophiocordyceps sinensis</em> in root tissue is explained by presence of <em>O. sinensis</em> in leaf tissue of the same plant, but not by local abundance of that plant and its vegetation type. Results of generalized linear mixed models with Bayesian estimations and phylogeometric random effects (500,000 iterations), tested with phylogeny at the level of sample species, genus and family.</th>
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<tbody>
<tr>
<td>leaf</td>
</tr>
<tr>
<td>species (n = 115)</td>
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<tr>
<td>genera (n = 57)</td>
</tr>
<tr>
<td>families (n = 27)</td>
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Fig. 1. (A) Hypothesized life-history of *Ophiocordyceps sinensis* as an endophytic insect pathogenic fungus (EIPF) whose dispersed ascospores infect the leaves of multiple families of host plants above the soil and move endophytically to plant root tissues, which are ingested by the herbivorous larvae of the ghost moth, *Thitarodes shambalaensis* (Lepidoptera: Hepialidae). (B) *Ophiocordyceps sinensis* endophytic presence in the leaf and root tissue of plant families (and phylum Bryophyta) found at the Haizidang caterpillar fungus collection site, and the presence of plant families (and phylum Bryophyta) in the guts of 73 larvae of *T. shambalaensis* collected at the site.
diet, was the only variable that showed a trend in level of infection for all samples, with older individuals more likely to show signs of severe infection (Fig. 4C, estimate $\text{size} = 0.25$, s.e. = 0.13, $p = 0.068$). From this, we suspect that the eight larvae collected in 2017 must have been harvested later in the season than those collected in 2018, which is why they all tended to be larger and showing a higher level of infection.

Variables related to the composition and diversity of an individual’s diet, such as phylogenetic diversity (PD), family level richness (SR) and phylogenetic signal (Fritz and Purvis’ D value), did not significantly predict its level of infection (i.e. having a low diversity diet, or a diet with high phylogenetic signal composed of plants of related lineages were not associated with a greater likelihood of infection). We also did not observe an individual’s diet to be correlated with any of the field traits we measured, such as the presence of *O. sinensis* in the roots, the vegetation type and the local abundance. Larvae whose diet consisted entirely of plant families also known to harbor *O. sinensis* showed no more signs of infection than those with a more diverse diet (Figs. 3 and 4, see Table S4 for individual data).

Comparing larvae in the two size classes, larger or smaller than 2 cm, we found no significant difference in average individual diet diversity (PD) and richness (SR) (Fig. 4A and B), nor was there a significant difference in their composition (PERMANOVA test on UniFrac distance matrix, $p_{\text{all samples}} = 0.486$, $p_{\text{2018 samples}} = 0.757$). Rarefaction curves of larval diet for both small and large larvae rapidly reach saturation (21 families eaten by small larvae and 19 families eaten by large larvae). Only two plant families collected in the habitat, Juncaceae and Solanaceae were not found in the guts of larvae, which are also known to harbor *O. sinensis* as an endophyte in both leaves and roots suggests that *O. sinensis* showed no more signs of infection than those with a more diverse diet (Figs. 3 and 4, see Table S4 for individual data).

Comparing the diets of the 11 most highly infected larvae with those of others, we found no significant difference in average individual diet diversity (PD) and richness (SR) (Fig. 4A and B), but there was a significant difference in their dietary composition (PERMANOVA test on presence/absence of high infection, UniFrac distance matrix, $p < 0.001$; MANTEL test on levels of infection, $p < 0.001$), as shown in the different trajectories of each group’s rarefaction curve (Fig. 4E).

### 4. Discussion

#### 4.1. *Ophiocordyceps sinensis* is a plant endophyte

Our results offer strong evidence that *O. sinensis* is not only a “classic” entomopathogenic fungus in the order Hypocreales, but also an endophytic insect pathogenic fungus (EIPF). The widespread prevalence of endophytic *O. sinensis* in Haizidang in 52.6% of all plant genera and 66.7% of all plant families (Table S1) is consistent with survey results from another caterpillar fungus habitat in Mt. Sejila, eastern Tibet Autonomous Region (Zhong et al., 2014), where more than half of the 42 plant species screened contained endophytic *O. sinensis*. The convergence of evidence from these two collection sites 670 km apart suggests that *O. sinensis* is a common plant endophyte in the natural habitats of caterpillar fungus.

We did not detect any evidence that endophytic *O. sinensis* might specialize on particular plant lineages. If *O. sinensis* had a plant-endophytic ancestor that was an obligate endophyte on particular plant lineages, this historical association has either been lost or was not detectable by the methods employed in this study. On the contrary, at both the genus and family level, endophytic *O. sinensis* is over-dispersed (Fritz and Purvis’ D larger than 1). Its association with multiple plant lineages likely reflects the relative ease of *O. sinensis* ascospores spreading indiscriminately over all plants present in a windy, high-elevation meadow habitat.

Our study uncovered a significant correlation between the presence of endophytic *O. sinensis* in plant leaf tissue and its presence in the root tissue of the same plant (Fig. 1, Table 1). EIPF are known to be localized in the plant tissues consumed by their insect hosts. For example, species of *Metarhizium* primarily infect soil-dwelling insects and are localized in plant roots, and *Beauveria* sp. primarily infect above-ground insects and are localized in plant leaves (Behie et al., 2015). Since *O. sinensis* infects only soil-dwelling *Thitarodes* larvae, it is perhaps surprising that endophytic *O. sinensis* are also found in plant leaf tissues.

We hypothesize that the presence of *O. sinensis* in plant leaf tissues indicates the initial site of contact between dispersing *O. sinensis* ascospores and their host plants. Further, the presence of *O. sinensis* as an endophyte in both leaves and roots suggests that after establishing on leaf tissue, it can readily translocate to the roots. The ease of leaf exposure to *O. sinensis* is supported by our observation that more leaf samples contained *O. sinensis* than did root samples. Using quantitative qPCR measurement, Lei et al. (2015) likewise showed that many plants had significantly higher leaf *O. sinensis* content than did their roots. It may be difficult for plant roots to encounter *O. sinensis* through the spread of mycelium in soil, although Yang et al. (1989) emphasized the role of precipitation in delivering ascospores in soil. The content of *O. sinensis* in soil is extremely low outside a 20 cm radius of a sporulating stroma (Peng et al., 2013). Even within the vicinity of a stroma, *O. sinensis* concentration is much higher on the soil surface and surrounding vegetation than under the soil (Peng et al., 2013). A leaf-to-root endophytic transfer of *O. sinensis* would be an excellent solution to an ecological dilemma: unlike most known EIPF (e.g. *Metarhizium* sp. and *Beauveria* sp.) that are generalist insect parasites, *O. sinensis* is host-specific, yet unlike other host-specific entomopathogenic fungi of the genus *Ophiocordyceps* that parasitize above-ground insects such as ants (Andersen et al., 2009), wasps (Shrestha et al., 2017) and flies (Xiao et al., 2019), the hosts of *O. sinensis* (larvae of *Thitarodes*) live exclusively underground. For *O. sinensis*, partnering with plants to reach a soil-dwelling root-nibbling host economizes an otherwise costly reproductive strategy. Our understanding of such an evolutionary strategy is limited, but could be illuminated by a controlled laboratory study to verify the “leaf-to-root transfer” hypothesis of endophytic *O. sinensis*, as well as a comparative study of the life histories of other species of Hypocreales fungi facing the same dilemma of hard-to-target obligate hosts. For example, species such as *Metacordyceps shibinensis*,

<table>
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<tr>
<th>leaf O. sinensis presence</th>
<th>root O. sinensis presence</th>
<th>vegetation type</th>
<th>local abundance</th>
<th>caterpillar consumption</th>
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<td>0.017 0.65</td>
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<td>0.352 0.5</td>
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<tr>
<td>families (n = 27) 1.69</td>
<td>0.087 0.92</td>
<td>0.258</td>
<td>0.291 1.26</td>
<td>0.17 0.16 0.33</td>
</tr>
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</table>

Table 2 Presence of *Ophiocordyceps sinensis* in plant tissues is an over-dispersed trait across the plant families at Haizidang caterpillar fungus habitat. We show the Fritz and Purvis’ D for phylogenetic signal of five binary traits, 1000 permutations, at three taxonomic levels: (1) O. sinensis presence in plant leaf, (2) O. sinensis presence in plant root, (3) vegetation type ("woody" or "herbaceous") (4) local abundance ("common" or "rare") and (5) consumption by larva. D is 1 if the distribution of binary trait is random, while D higher than 1 suggests over-dispersion of trait. D is 0 if the binary trait shows strong phylogenetic signal. P-value is the probability of obtaining the calculated Fritz and Purvis’ D from a Brownian phylogenetic structure.
Cordyceps gunnii and Cordyceps liangshanensis are all host-specific parasites of soil dwelling lepidopteran larvae (Sung et al., 2007; Wen et al., 2015). In these cases, we would predict a convergent mechanism whereby these fungi might function as EIPF, infecting the leaves of their host plants before travelling to the roots where they could gain access to a root-eating caterpillar.

4.2. Larval gut content

Absence of a significant phylogenetic signal for the plant families consumed by Thitarodes larvae (Table 1) suggests that larvae are generalist root eaters, and these data are consistent with previous studies on the dietary preferences of Thitarodes larvae.
Fig. 3. Principal components analysis (PCA) plot showing the axes of variation of the family level dietary composition of each larva. The arrows represent major plant families that explain the differences in larval diet. Each larval diet is indicated by a circle. The darker color fills indicate higher estimated level of *O. sinensis* fungal infection observed in those larvae, while the empty circles indicate the larvae had no or low-level infection. The size of each circle indicates the phylogenetic diversity (PD) of the larva’s plant diet. For each larval diet, we tested whether there were phylogenetic associations with plant lineages that have root endophytic *O. sinensis*. The only two samples that showed a significant correlation are labeled in red circles, but these two samples did not show high level of *O. sinensis* infection.

Fig. 4. Relationship between diet content, larval size of *Thitarodes shambaraensis* ghost moth (n = 73), and level of infection by *Ophiocordyceps sinensis*. (A) Diet phylogenetic diversity (PD) does not differ significantly between larger larvae (“big”; >2 cm) and smaller ones (“small”; ≤2 cm) or between larvae with high estimated levels of infection (“high”) and those with low-level or no infection (“low”). (B) Diet richness (SR, number of plant families detected in the diet) did not differ between big and small larvae, or between larvae with high or low levels of infection. (C) Estimated infection level is correlated with larval size (estimate = 0.50, s.e. = 0.11, p < 0.001). (D) Rarefaction curve of diet richness between larvae larger and smaller than 2 cm. (E) Rarefaction curve of diet richness between larvae with high or low levels of *O. sinensis* infection.
(Table S5). Larvae in natural habitats have been documented to have diets with a higher proportion of plants in family Polygonaceae (\textit{Polygonum viviparum}, \textit{Polygonum sphaerochostachyum}, \textit{Rheum pumilum}) and Ranunculaceae (\textit{Ranunculus tanguticus}, \textit{Caltha scoposa}), although whether this is because the roots of these plants are more abundant in the habitat or because the larvae select them for some other reason is not known (Yin et al., 1995). In captive breeding experiments, plants in the family Poaceae (malt and millet sprout) are used to feed larvae (Tao et al., 2015). In our study, these three families (Polygonaceae, Ranunculaceae, Poaceae) are the vascular plant families with the highest OTU counts and the highest presence/absence counts detected in larval gut content. Of the 12 families of plants described as host plants of \textit{Thitarodes} larvae, 11 were detected in the larval guts analyzed in this study (Yin et al., 1995; Zhou et al., 2014; Tao et al., 2015, summarized in Table S5). Our study is the first to detect bryophytes, both by OTU counts and by OTU presence, in \textit{Thitarodes} guts as prevalent.

The significant correlation between an individual larva’s estimated level of infection with its size most likely reflects the observation that \textit{O. sinensis} infection has its onset in the 4th and 5th instar larvae that are larger than 2 cm long (Yang et al., 1989). Although the diversity and specificity of individual larval diets vary across individuals (Table S4, Fig. 3, Fig. S3), none of these diet-related parameters significantly predicted the level of \textit{O. sinensis} infection.

Our study confirmed that plant families harboring \textit{O. sinensis} in their roots are consumed by \textit{Thitarodes} larvae. Although larvae do not appear to prefer phylogenetically related groups of host plants, we note that all the plants with some level of infection (leaf only, both or root only) had strikingly higher levels of infection than plants that were not consumed. At its most extreme, 100% of those plant families (including bryophytes) where both leaves and roots were infected by \textit{O. sinensis} (n = 10) were consumed by larvae of \textit{T. shambalaensis}, whereas only 67% of completely uninfected plant families (n = 9) were consumed (Chi-squared = 1.85, p = 0.174). This trend in consumption of the roots of plants that have been infected by \textit{O. sinensis} was not statistically significant, but given that we sampled the plants at only one site, and the diet was characterized for only 73 caterpillars, it suggests that \textit{O. sinensis} might somehow make the roots that it inhabits more attractive to larvae of \textit{T. shambalaensis}. The release of allelochemicals into the rhizosphere that mediate the interactions between plant roots, bacteria, fungi and/or invertebrates is well known (e.g. Wenke et al., 2010). It seems plausible that a parasite such as \textit{O. sinensis} might recruit its secondary host in this way, and this could be tested experimentally in future studies by comparing the attractiveness of infected versus uninfected root tissue to larvae of \textit{T. shambalaensis}.

Further evidence also suggests that root endophytic \textit{O. sinensis} ingested by larvae is the source of larval infections. First, qualitative observation and quantitative assessment of \textit{O. sinensis} concentration inside infected \textit{Thitarodes} larvae showed that in some samples, the concentration of \textit{O. sinensis} was higher in larval guts than on the surface of the larval exoskeleton (Lei et al., 2015; Li et al., 2016). Second, stable carbon isotope analysis demonstrated a decrease of δ¹³C values from the larval head to the abdomen, but remained constant along the abdomen, suggesting initiation of fungal growth at the head of the larvae and a stable growth environment along the digestive tract during infection (Guo et al., 2017). Third, as previously mentioned, \textit{O. sinensis} concentration in the soil is low, making larval infection through exoskeleton contact with soil mycelium unlikely. \textit{Ophiocordiceps sinensis} is, therefore, likely partnering with plants to gain access to its soil-dwelling host via ingestion of the host plant roots. This scenario does not exclude the possibility that \textit{O. sinensis} in soil can infect its host through penetration of host exoskeleton. We also note that endophytic fungi in roots can further release spores into the rhizosphere and infect larval hosts in the vicinity. Yang et al. (1989) observed significant correlation between increase in \textit{Thitarodes} infection rate and increase in \textit{Thitarodes} feeding activities.

5. Conclusions

We show that the entomophagous caterpillar fungus, \textit{O. sinensis}, is a widespread plant endophyte in the high elevation meadows of the Himalaya–Hengduan regions where “caterpillar fungus” is harvested by local people for its medicinal properties, and that the presence of \textit{O. sinensis} in the leaf tissue of a plant species is significantly correlated with its presence in the root tissue of the same plant species. Our analysis of the diets of caterpillars of ghost moth larvae, \textit{T. shambalaensis}, confirms that plant families with endophytic \textit{O. sinensis} are also consumed by \textit{T. shambalaensis} larvae, although we did not detect a significant correlation between the composition and diversity of an individual larva’s diet and its level of fungal infection. Our results suggest that \textit{O. sinensis} has a reproductive strategy that involves an endophytic life stage in plants that facilitates infection of host larvae through consumption of infected root tissue. Larvae appear to be more likely to consume plant families that also harbor \textit{O. sinensis}, suggesting that infected host plant roots may be more attractive than uninfected roots to larvae of \textit{T. shambalaensis}. The overlooked, third party role of plants in this insect-fungus symbiosis may help to explain some of the difficulties that have been encountered in efforts to artificially infect larvae of several species of \textit{Thitarodes} for commercial cultivation of caterpillar fungus. Our study also highlights the importance of the diverse habitat vegetation to the growth of caterpillar fungus. Preservation of caterpillar fungus must focus on preservation of the habitat, including its vegetation.

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Supplementary data

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