Fungal Ecology 47 (2020) 100989

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

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

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ARTICLE INFO

Article history:
Received 5 June 2020
Received in revised form
4 August 2020
Accepted 9 August 2020
Available online xxx

Corresponding Editor: Nicolai Vitt Meyling

Keywords:
Endophytic insect pathogenic fungi
EIPF
Entomophagous fungi
Ophiocordyceps sinensis
Thitarodes shambalaensis
Mt. Gongga
Diet
Gut content

ABSTRACT

Endophytic insect pathogenic fungi of the order Hypocreales reside in plant tissues as mutualistic partners that protect plants against insect herbivores. Caterpillar fungus *Ophiocordyceps sinensis* (order: Hypocreales, family: Ophiocordycipitaceae) is often described as an entomophagous fungus that parasitizes lepidopteran larvae of the ghost moth genus *Thitarodes* (family: Hepialidae), but recent evidence suggests involvement of a plant-endophytic stage in *O. sinensis* infection of insect larvae. In this study, we screen sample plant species in a caterpillar fungus collection site at Mt. Gongga, Sichuan, China, for endophytic *O. sinensis* and analyze the diet of *Thitarodes* larvae living in the soil of the same habitat. We show that the entomophagous caterpillar fungus *O. sinensis* is a prevalent plant endophyte (present in 52.6% of all plant genera and 66.7% of plant families) and that the presence of endophytic *O. sinensis* in plant leaf tissue is significantly correlated with its presence in plant root tissue. Our analysis of larval diet content reveals a high diversity generalist diet and confirms that the plant families harboring endophytic *O. sinensis* are consumed by *Thitarodes* larvae, although the composition and diversity of an individual's diet do not predict its level of fungal infection. These results suggest a reproductive strategy of *O. sinensis* involving a plant endophytic stage that facilitates infection of root-eating host larvae. Our study highlights the role of resident plant communities as a third participating partner in this complex symbiosis.

1. Introduction

Endophytic insect pathogenic fungi (EIPF) are insect-infecting 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 Branine et al., 2019 for review). EIPF use homologous genes in the penetration of both plant cell walls and insect

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https://doi.org/10.1016/j.funeco.2020.100989
1754-5048/

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 (Spatafora et al., 2007), but recent phylogenomic analyses suggest the common ancestor of the order Hypocreales is plant pathogenic, experiencing cross-kingdom host shifts from plant 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 hypocrealean fungi (Zhang et al., 2018). These data suggest that hypocrealean fungi currently described as solely entomopathogenic might also be capable of a plant-endophytic lifestyle.

Ophiocordyceps sinensis (order: Hypocreales, family: Ophiocordycipitaceae) is often described as an entomophagous fungus that parasitizes lepidopteran larvae of the ghost moth genus *Thitarodes* (family: Hepialidae) (Sung et al., 2007; Shrestha et al., 2014). *Ophiocordyceps sinensis* 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 et al., 2018). Different *O. sinensis* strains and their *Thitarodes* hosts exemplify a co-diversified system of insect-fungus symbiosis, involving at least 7 fungal clades and as many as 54 *Thitarodes* moth species (Zhang et al., 2014; Quan et al., 2014a, b).

Compared with the wealth of information on the evolutionary history and socio-economic impact of this entomophagous fungus, relatively little is known about the mechanism by which *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 *Tolypocladium* 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 fungus 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 one 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; García-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 (Boufford 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 km^2 in Yanzigou valley, Mt.

Gongga (29°40′17.18″N, 101°53′48.25″E; alt. 3977 m; hereafter, we refer to this site as "Haizidang" for brevity). Villagers on the eastern side of Mt. Gongga have been collecting at this locality for at least two decades. This site is the type locality of *Thitarodes shambalaensis* (Wang et al., 2019b), a newly identified ghost moth host of *O. sinensis*. The studies presented here took place over three field seasons in 2017, 2018 and 2019, after most *O. sinensis* had formed ascomata and dispersed ascospores. All plants in the site could be identified to genus, but not to species, with the exception of bryophytes, which we lumped together as a single monophyletic sampling unit during our analysis. We, therefore, defined our sampling units as morpho-species (designated as a "sample species"). We sampled leaf and root tissue from all observed plant species throughout the site. We collected three replicates of each sample species, at least 20 m apart from each other. To obtain a binary estimate of the local abundance of each sample species, we marked a sample species as locally "rare" if we had to search for more than 100 m to obtain a second replicate of the same sample species (or if less than 3 replicates could be obtained); otherwise, a sample species was marked as locally "common". The vegetation type of each sample species was noted as either "woody" or "herbaceous". The leaf and root tissues of each replicate of each sample species were separately preserved and transported to the lab for DNA extractions. All samples were washed in 70% ethanol and surfaced-sterilized with sodium hypochlorite. After grinding, DNA was extracted using a Tiangen Plant Genomic DNA kit. Quantitative PCR with primers designed to detect *O. sinensis* was conducted following the protocol of Lei et al. (2013) (Bio-Rad CFX96, SYBR Green PCR Master Mix, three replicate reactions per sample). A caterpillar fungus sample collected at Haizidang and characterized using the same extraction protocol was used as a positive control. The first two positive qPCR detections were sequenced to verify *O. sinensis* identity. A sample plant species was regarded as containing *O. sinensis* endophyte (in its leaf or root) if *O. sinensis* was detected in at least one of the three field-collected sample replicates (of either leaf or root).

2.2. Larval diet

At Haizidang, *T. shambalaensis* larvae were sampled by randomly excavating 1 m^2 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 ($\leq$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 each sample was dissected and preserved in 90% ethanol. DNA from the contents of each mid-gut were extracted with a Tiangen Genomic DNA kit. A fragment of the large subunit of the ribulose bisphosphate carboxylase (*rbc*L) 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.

2.3. Analysis

To construct a phylogeny representing the diversity of plants at Haizidang, we either sequenced the *rbc*L region of the sample plant species, or assigned the sample species to an *rbc*L sequence of the same genus from GenBank (Table S3). With the exception of two bryophyte samples, all other samples were identified to the genus level. Our sequences were aligned in Geneious (version 2019.0.4) with MUSCLE alignment (Edgar, 2004). A maximum likelihood tree was built on CIPRES Science Gateway (Miller et al., 2010) using RAxML-HPC v.8, GTRCAT model approximation (Stamatakis, 2014). To ensure that our genus-level phylogeny will have the most up-to-date family-level topology, we trimmed the family-level vascular plant phylogeny of Li et al. (2019) and used it as the topological constraint for our RAxML run, while using our bryophyte *rbc*L sequence as an outgroup.

Binary scores were entered for the following traits for each plant sample: (1) endophytic *O. sinensis* presence/absence in sample root, (2) endophytic *O. sinensis* presence/absence in sample leaf, (3) vegetation type ("woody" or "herbaceous"), (4) local abundance ("common" or "rare") and (5) plant family represented in larval gut ("present" or "absent", from larval gut content analysis, see below). To assess the phylogenetic signal of these traits, we applied the Fritz and Purvis (2010) test in the R package "*caper*" (Orme et al., 2018) to test whether these traits are randomly distributed (i.e. no phylogenetic signal), over-dispersed or conserved with respect to the phylogeny. We conducted the test at the level of each sample species, each genus and each family. If a single family contained samples of both vegetation types, and both levels of local abundance, we scored it as having the less common trait types (i.e. "woody" and locally "rare") to maximize the ability to detect phylogenetic signal. To test whether any of these traits are associated with each other (e.g. whether presence of *O. sinensis* in the roots is associated with presence of *O. sinensis* in the leaves), we constructed a generalized linear mixed model with Bayesian estimations, using phylogenetic variance-covariance as a random effect (500,000 iterations, inverse-Gamma distribution prior) (Hadfield, 2010, R package "*MCMCglmm*" in R).

Since plant primers used for this study yield amplicon sequences that are most reliably identified to the family level (Hofreiter et al., 2000), plant OTUs identified from larval gut contents were taxonomically assigned to the family level (although all bryophyte OTUs were analyzed as a single taxonomic unit). We filtered the family-level OTU table with the families of plants we collected at Haizidang. The family-level OTU counts were converted to a presence/absence matrix in all analyses to reflect the detection of different plant families (Deagle et al., 2019). Phylogenetic diversity (PD), species richness (SR) and rarefaction curves of each larval diet were calculated in the R package "*vegan*" (Oksanen et al., 2013).

From the family-level plant OTU presence/absence table of all larval gut contents, we analyzed the phylogenetic signal of each individual larval diet by conducting the Fritz and Purvis (2010) test per dietary composition, using the family level plant phylogeny and presence/absence as a binary trait. To look at the association of each individual larval diet with plant traits measured in the field (*O. sinensis* endophyte in root, vegetation type, local abundance), we applied Phylogenetic Generalized Least Squares analysis (Ives et al., 2007, implemented in R package "*phytools*", Revell, 2012), treating response variables as continuous. To analyze the variables associated with the estimated infection level of each larva, we ran a generalized linear model (R package "*glm*") with six variables: (1) the size of the larva, (2) the phylogenetic diversity (PD) of its diet, (3) family-level "species" richness of its diet (SR, i.e. number of families detected in diet), (4) the phylogenetic correlation of its diet

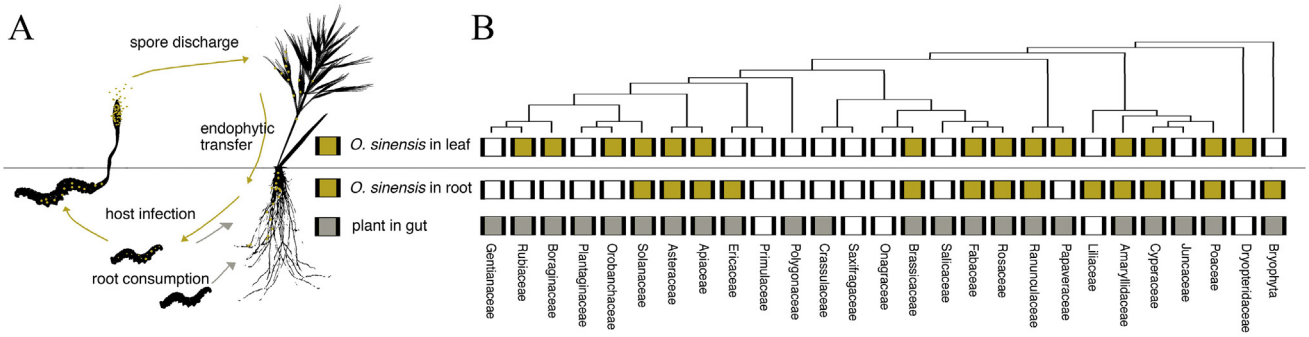

Fig. 1. **(A)** Hypothesized life-history of *Ophiocordyceps sinensis* as an endophytic insect pathogenic fungus (EIPF) whose dispersed ascospores infect the leaves of multiple families of host plants above the soil and move endophytically to plant root tissues, which are ingested by the herbivorous 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.

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 categorial 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 unidentified 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 *rbc*L 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 ($DE_{max} = 2.59$, $DE_{min} = -3.75$, 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. S3).

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
Presence of *Ophiocordyceps sinensis* in root tissue is explained by presence of *O. sinensis* 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 phylogenetic random effects (500,000 iterations), tested with phylogeny at the level of sample species, genus and family.

	leaf	p	vegetation type (wood)	p	local abundance (rare)	p
species (n = 115)	0.35	<0.001	−0.04	0.76	0.03	0.709
genera (n = 57)	0.38	0.008	0.08	0.721	−0.05	0.725
families (n = 27)	0.48	0.016	0.38	0.244	0.07	0.697

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 larvae. 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.

	leaf *O. sinensis* presence	p	root *O. sinensis* presence	p	vegetation type	p	local abundance	p	caterpillar consumption	p
species (n = 115)	0.94	<0.001	1.01	<0.001	0.5	0.017	0.65	<0.001		
genera (n = 57)	1.17	<0.001	1.67	<0.001	0.26	0.352	0.5	0.056		
families (n = 27)	1.69	0.087	0.92	0.258	0.84	0.291	1.26	0.17	0.16	0.33

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 $_{size} = 0.25$, 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 a there significant difference in their composition (PERMANOVA test on UniFrac distance matrix, $p_{all\_samples} = 0.486$, $p_{2018\_samples} = 0.757$). 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 large larvae (Fig. 4D). 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" entomophagous 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 (*Metarhizium* sp. and *Beauveria* sp.) that are generalist insect parasites, *O. sinensis* is host-specific, yet unlike other host-specific entomophagous fungi of the genus *Ophiocordyceps* that parasitize above-ground insects such as ants (e.g. 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*,

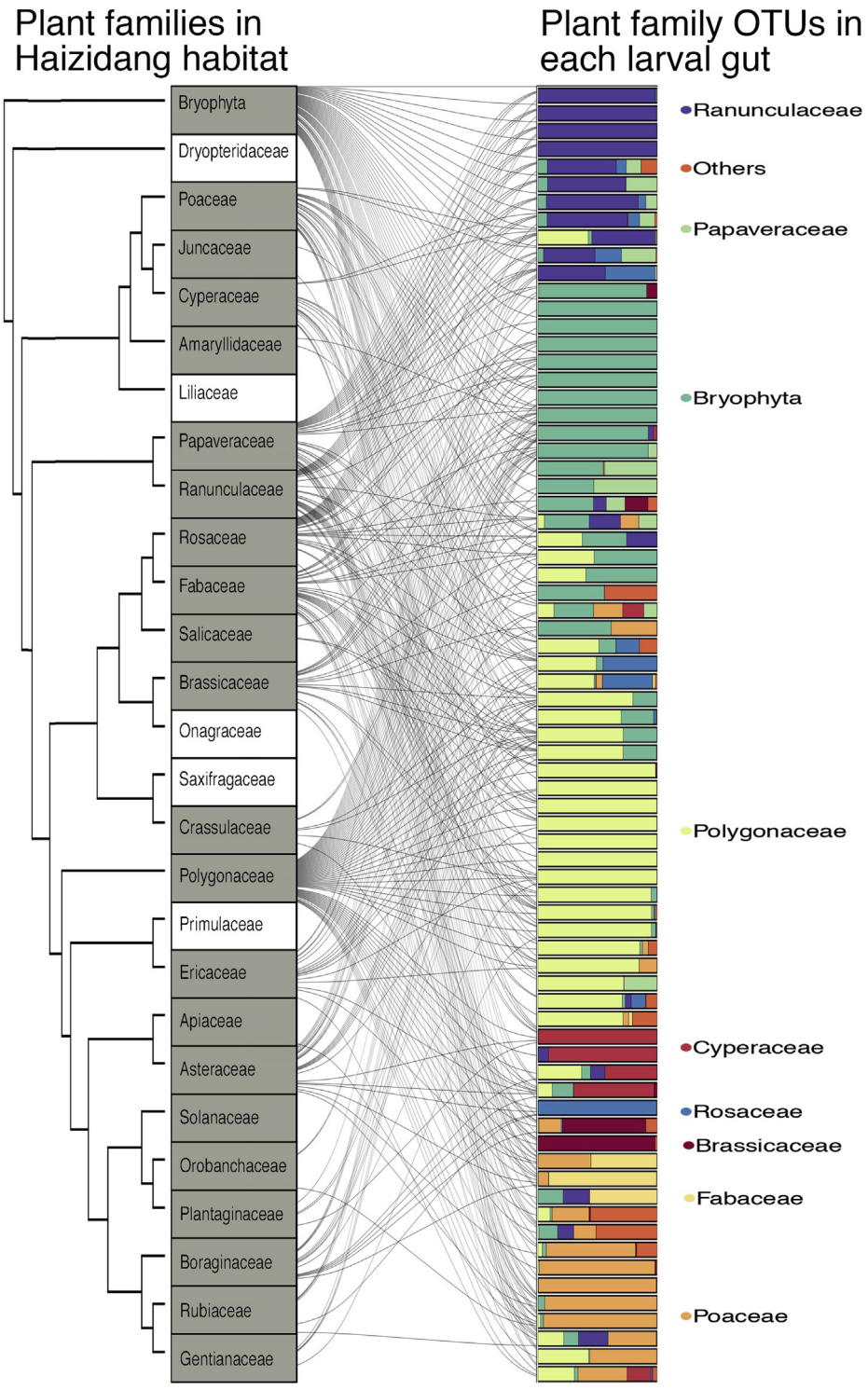

Fig. 2. Diet of 73 larvae of *Thitarodes shambalaensis* collected at the Haizidang caterpillar fungus collection site. On the left, a family level phylogeny of potential host plants present in the habitat (including the phylum Bryophyta), with grey bars indicating consumption by larvae. On the right, colored bars indicate the percentage of family level OTU counts for each larval gut.

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

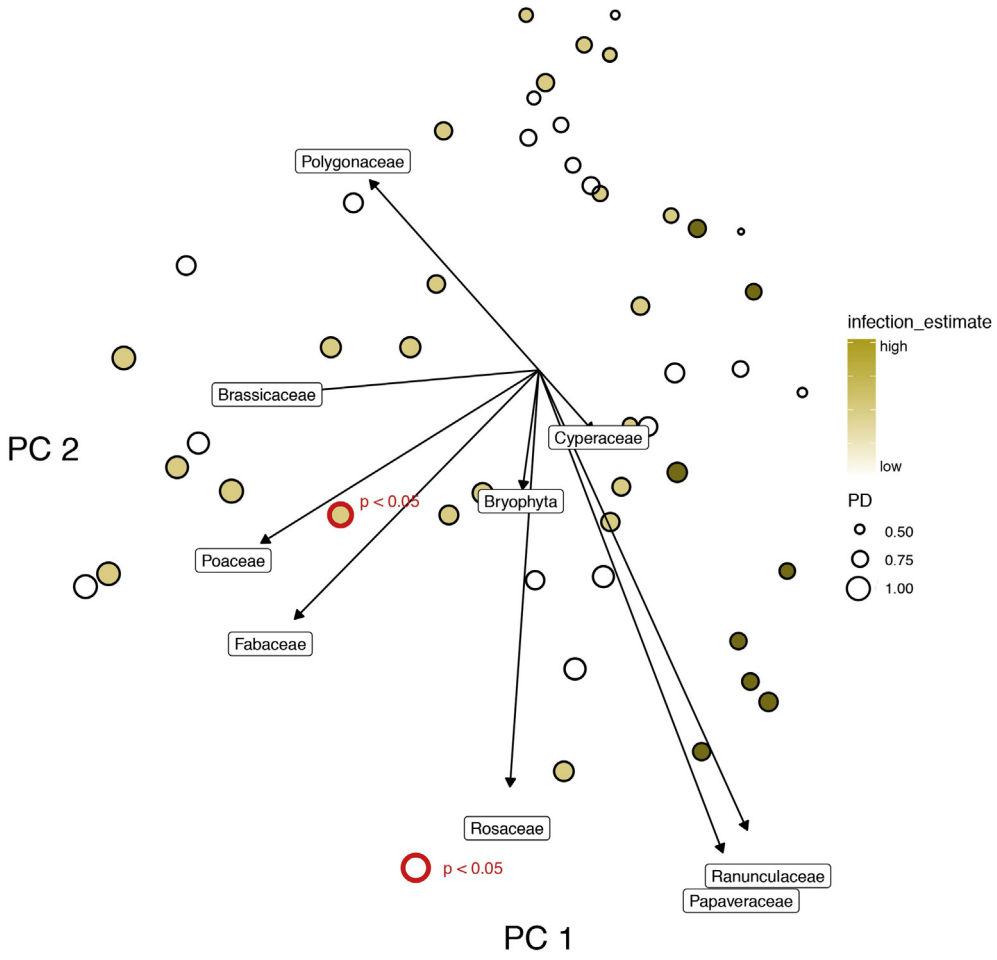

Fig. 3. Principal components analysis (PCA) plot showing the axes of variation of the family level dietary composition of each larvae. The arrows represent major plant families that explain the differences in larval diet. Each larval diet is indicated by a circle. The darker color fills indicate higher 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 larvae'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.

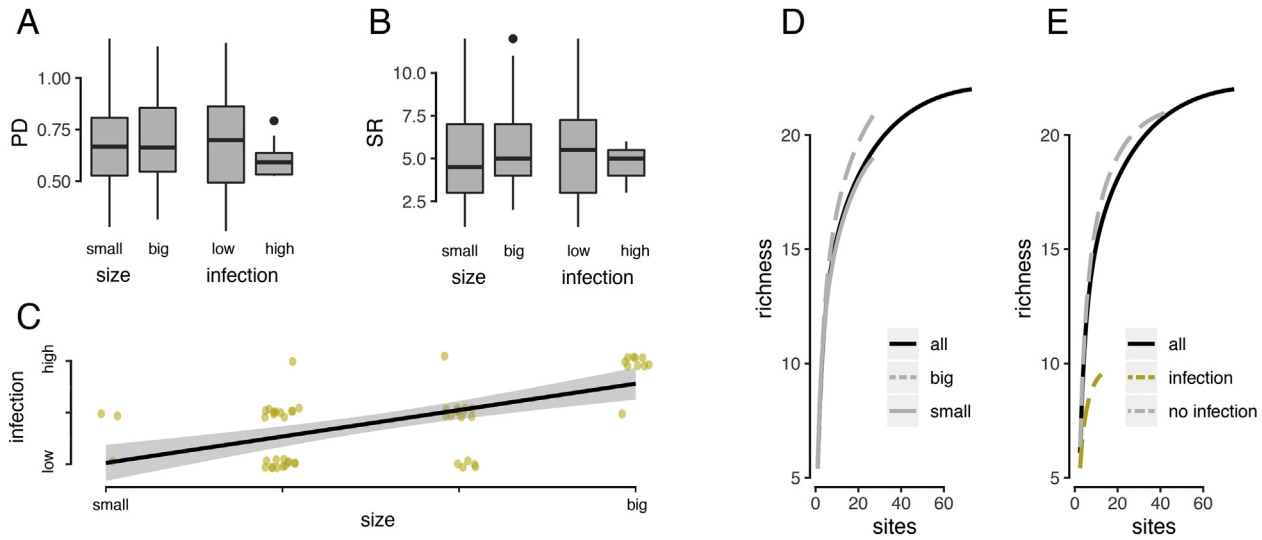

Fig. 4. Relationship between diet content, larval size of *Thitarodes shambalaensis* ghost moth (n = 73), and level of infection by *Ophiocordyceps sinensis*. **(A)** Diet phylogenetic diversity (PD) does not differ significantly between larger larvae ("big"; >2 cm) and smaller ones ("small"; ≤2 cm) 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 (*Polygonum viviparum, Polygonum sphaerostachyum, Rheum pumilum*) and Ranunculaceae (*Ranunculus tanguticus*, *Caltha scaposa*), 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 *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 *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 *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 *O. sinensis* infection.

Our study confirmed that plant families harboring *O. sinensis* in their roots are consumed by *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 *O. sinensis* ($n = 10$) were consumed by larvae of *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 *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 *O. sinensis* might somehow make the roots that it inhabits more attractive to larvae of *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 *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 *T. shambalaensis*.

Further evidence also suggests that root endophytic *O. sinensis* ingested by larvae is the source of larval infections. First, qualitative observation and quantitative assessment of *O. sinensis* concentration inside infected *Thitarodes* larvae showed that in some samples, the concentration of *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 $\delta^{13}C$ 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, *O. sinensis* concentration in the soil is low, making larval infection through exoskeleton contact with soil mycelium unlikely. *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 *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 rhizospheres and infect larval hosts in the vicinity. Yang et al. (1989) observed significant correlation between increase in *Thitarodes* infection rate and increase in *Thitarodes* feeding activities.

5. Conclusions

We show that the entomophagous caterpillar fungus, *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 *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, *T. shambalaensis*, confirms that plant families with endophytic *O. sinensis* are also consumed by *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 *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 *O. sinensis*, suggesting that infected host plant roots may be more attractive than uninfected roots to larvae of *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 *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.

Acknowledgements

We thank villagers from Yanzigou valley: Huailiang Tang, Youjun Tang, Zulian Zhou and Huaping Zuo for their field assistance and hospitality throughout the years during which we have conducted field work. Without their help and assistance, this research would have been logistically impossible. We thank Kadeem Gilbert, Cong Liu and Fuli Zhang for numerous helpful discussions during the analysis, and Sofia Prado-Irwin for commenting on an early version of the manuscript. We thank our subject editor Nicolai Vitt Meyling and two anonymous reviewers for greatly improving the scientific rigidity of the manuscript. ZW is supported by a graduate fellowship from Harvard University Department of Organismic and Evolutionary Biology.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2020.100989.

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