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Transmission of symbiotic fungus with a nonsocial leaf-rolling weevil

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ABSTRACT

Transmission modes of symbionts with fungus-growing insects are closely related to the stability of symbioses. As compare to social fungus-farming insects, transmission modes of nonsocial fungus-farming insects need to be further investigated. *Euops chinensis*, a nonsocial leaf-rolling weevil, harbors a symbiotic fungus *Penicillium herquei* in the specialized mycangium. Previous works have indicated that *P. herquei* is cultivated to host plants by females during oviposition process, however, it is still unclear when (before or after oviposition) and how *P. herquei* is transmitted. In this study, we observed fungal cultivating behaviors and adult bodies by scanning electron microscopy (SEM), and compared isolation rates of *P. herquei* on ovary eggs, newly oviposited eggs, cradle leaves (leaf pieces cut before rolling cradles), cradles, and female mycangia. Fungal isolates were identified by internal transcribed spacers (ITS) and cytochrome oxidase I (COI) genes. We found the female's serrated tarsi and comb-like setae on the abdomen were specialized structures for fungal transmission. Newly oviposited eggs showed 81.11% frequency of fungal symbionts, but ovary eggs did not show any growth of fungal symbionts. Isolation rates of *P. herquei* on cradle leaves, mycangia and cradles were 76.67%, 77.71% and 87.72%, respectively. Analyses of ITS and COI genes showed that isolated fungal strains belong to the same species. We concluded that *P. herquei* was transmitted before oviposition, and the female's tarsi are newly found specialized structures for fungal transmission. This study elucidates the cultivar transmission mode with fungus-farming attelabid weevils, and might be useful to study of other fungiculture mutualisms.

Introduction

Mutually beneficial symbioses between insects and microbes are ubiquitous in nature, and it also plays an important role as vector of evolutionary novelty and ecological diversity (Janson et al., 2008; Kawaguchi, 2011; Six and Klepzig, 2004). In many cases, insects develop the capacity to cultivate fungi (Kobayashi et al., 2008; Mueller et al., 2005; Toki et al., 2012), and are dependent on their microbial associates for nutrition, defense, or development (Ayres et al., 2000; Douglas, 2009). Instances of fungiculture have been not only found in some social insects such as leaf-cutting ants (Currie et al., 2006), ambrosia beetles (Endoh et al., 2011), and termites (Aanen et al., 2002), but also in some non-social insects like wood wasps (Srutka et al., 2007), tag beetles (Tanahashi et al., 2010), and animals like marine snails (Silliman and Newell, 2003). Therefore, forming persistent associations with microbes is considered as an important evolutionary advantage for insect nutrition and physiological ecology (Douglas, 2009). However, fungiculture of social insects are relatively well-documented as compared to non-social insects (Sakurai, 1985; Silliman and Newell, 2003; Toki et al., 2012).

The symbiont transmission modes with insects validates the competence and stability of symbiosis (Bright and Bulgheresi, 2010), and making it one of the most unique processes for maintenance of symbiotic associations (Hosokawa et al., 2007; Korb and Aanen, 2003). Insects either be able to acquire a symbiont from the environment (horizontal transmission) (Kaltenpoth et al., 2009), or inherit it from their parents (vertical transmission)(Hosokawa et al., 2013). Among them, uniparental vertical transmission (via one of the two sexes) appears to be predominant in insects (Herren et al., 2013; Hosokawa et al., 2007; Korb and Aanen, 2003). Many fungiculture insects have evolved special exoskeleton cavities (called "mycangia") on their bodies (Grebennikov and Leschen, 2010; Tanahashi et al., 2010; Toki et al., 2012). Mycangia function as reservoirs for fungal spores or conidia (Grebennikov and Leschen, 2010), and are considered to be able to facilitate the dispersal (Six and Klepzig, 2004) and the homogeneity of symbionts (Brownlie and Johnson, 2009; Scott et al., 2008).

A seldom-studied example of fungiculture exhibited by non-social insects is leaf-rolling weevils (Coleoptera: Attelabidae) (Sakurai, 1985; Wang et al., 2015). Those weevils show special characteristics that

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adults manipulate the host plants and construct sophisticated cradles for their larvae (Liang and Li, 2005). Among the subfamily Attelabinae, the species in the *Euops* genus form a symbiotic association with fungi (Kobayashi et al., 2008; Li et al., 2012; Sakurai, 1985). The symbiotic fungi are carried in special mycangia by the female weevils (Kobayashi et al., 2008; Li et al., 2012), and can assist the development of the weevils by altering chemical components of leaf substrates and protecting against pathogens (Kobayashi et al., 2008; Li et al., 2012). Sakurai (1985) has reported that the attelabid weevil *Euops splendidus* possesses a set of fungus-cultivating structures including spore reservoir, spore incubator, and spore bed to incubate fungal spores. Although symbiotic fungi are known to be cultivated to plants during oviposition process (Sakurai, 1985; Wang et al., 2015), how and when (before or after oviposition) that the fungal cultivars are transmitted are still unclear.

Here, we investigated the intergenerational transmission modes of *Penicillium herquei* cultivated by its host *Euops chinensis* (Coleoptera: Attelabidae)(Liang and Li, 2005), a leaf-rolling weevil feeding on Japanese knotweed *Fallopia japonica* (Houtt.) Ronse Decraene (Wang et al., 2010). *P. herquei* is harbored in a special mycangium of the female adults (Li et al., 2012; Wang et al., 2010), and can produce the antibiotic (+)-scleroderolide to protect the fungal niches against potential infection (Wang et al., 2015). The female weevil cuts a narrow strip (we call 'cradle leaf') from the whole leaf, then nibbles and rolls it, finally deposits one eggs in each cradle (leaf-roll) (Li et al., 2012; Wang et al., 2015). Yellow mycelia of *P. herquei* usually cover cradle during a weevil developing through egg, larva, pupa, adult stages in the cradle. Whereas the leaves of *F. japonica* that are not rolled by *E. chinensis* harbor many different microbes except for *P. herquei* (Wang et al., 2015). Therefore, we hypothesized that *P. herquei* is probably transmitted with the female weevils before oviposition. We observed cradle constructing behaviors and the ultrastructure of adult bodies, and compared isolation frequencies of *P. herquei* on the eggs, cradle leaves, cradles, and mycangia of adults. Two DNA barcode markers (the nuclear internal transcribed spacers (ITS) of the ribosomal repeats, and the mitochondrial cytochrome oxidase I (COI) locus) were used for species-level identification of these fungal symbionts. We wanted to determine: 1) *P. herquei* is transmitted from adults to plants before or after oviposition? 2) Which structures are used for fungal transmission?

Materials and methods

Collection of insects and cradles

Insects (overwintered adults) and cradles samples from *F. japonica* were collected from Mt. Mingyue (MY) (N27°46′16.33, E114°23′38.30) in Jiangxi Province, China, in early May 2012. Cradles samples were put individually into sterilized tubes, and adults were maintained in the two different cages (20 cm × 13 cm) with live *F. japonica* seedlings under natural conditions at the Wuhan Botanical Garden (WBG) (N30°32′32.08, E114°2324′50.01), Hubei Province, China. The climate of Wuhan in summer is just similar to Jiangxi Province (both have a subtropical humid monsoon climate with hot and humid summers, the average temperature and relative humidity in summer is about 33 °C and 75%, respectively) (Zhang, 1988). Plant materials were watered every day for adults to construct cradles.

Behavioral and morphological observations

We closely observed cradle constructing behaviors of the females. Meanwhile, scanning electron microscopy (SEM) was used to detect the localization of fungal symbionts on adults of *E. chinensis*. Weevil adults were firstly sexed using a hand lens, just the females having six rows of erect pubescences on the basal sternite (Sakurai, 1985). Three females and three males were killed with 50 mL 100% diethyl ether, and processed according to the method of Yuceer et al. (2011). The specimens were firstly dehydrated through a graded ethanol series (25%, 50%, 75%, 80%, 90%, and 100%, 15 min at each concentration, with two repetitions at each ethanol concentration). All tissues were dried properly and coated with gold palladium alloy using a JFC 1600 auto fine sputter coater (JEOL, Japan). Samples were observed with a Quanta 2000 SEM (FEI, America).

Isolation of microorganisms from ovary eggs and newly oviposited eggs

To examine whether *P. herquei* is transmitted to the eggs during they are developing in the ovary or during oviposition process, we isolated fungal symbionts form ovary eggs and newly oviposited eggs. We killed 10 mature females with 50 mL 100% diethyl ether, and the mature eggs were stripped from the ovaries with a microscope (Olympus BX 51, Olympus, Japan) under 10× magnifications. Two or three randomly selected eggs from each ovary were placed separately on a potato dextrose agar (PDA) plate. A total of 72 eggs were examined from 30 ovaries (three replicates). Besides, 30 newly oviposited eggs were carefully removed from cradles, and each egg was placed separately to PDA plates. This procedure was also repeated three times. All the plates were maintained in an incubator at 25 °C for 5 days. After incubation, isolated fungal colonies were purified and stored on PDA slant at 4 °C for further use.

Isolation of microorganisms from cradle leaves, cradles and female mycangia

To find out whether *P. herquei* is inoculated onto the plant leaves before or after oviposition stage, we isolated symbiotic microorganisms from cradle leaves, cradles and female mycangia. When the female adults began to construct cradles, thirty cradle leaves, 30 cradles and 30 mature females were randomly collected from field. Each leaf tissue was cut into 3–5 small pieces (2 mm) and placed on PDA plates. Fungi associated with cradles were isolated according to the method of Li et al. (2012). Briefly, after eggs were removed from cradles, each cradle tissue was cut into 3 to 5 pieces and placed on a sterilized PDA medium plate. Fungal spores in mycangia were directly removed with a sterile needle and streaked onto PDA plates. These procedures were repeated 3 times. All the plates were maintained in an incubator at 25 °C for 5 days, and fungal cultures were purified and stored as above.

Identification of fungal symbionts

After the isolation, all the fungal symbionts were initially characterized based on morphological characteristics (Li et al., 2012; Wei, 1979), and in morphological observation, we found that all isolates have similar kinds of characters and it is not easy to find difference among the isolates.

To further identify the fungal strains, two genes ITS and COI were used in present study. Fungal DNA was extracted from 200 mg of each material according to manufacturer's instructions of the Tiangent DNA Mini Kit (Beijing, China) and ITS region was amplified by the primers ITS l and ITS 4 (White et al., 1990), while primers Pen F1 and AspR1 were used for amplification of COI (Seifert et al., 2007). Both amplifications were carried out in 25 μL reaction systems, and the PCR profile for the ITS region was the same as for Li et al. (2012). The PCR amplification for the COI gene was conducted as follows: initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 90 s, followed by a final extension at 72 °C for 10 min, finishing with 10 °C. PCR products were sequenced by Sangon Biotech Co. Ltd. (Shanghai, China) using an ABI 3730 Automated DNA Sequencer.

Multiple alignments of unknown sequences with reference sequences were performed in DNAMAN. A neighbor-joining (NJ) tree was created (Saitou and Nei, 1987) and clustered using the MEGA 5 package (Tamura et al., 2011) with a bootstrap value of 1000 replicates.

Statistical analyses

The isolation rate of *P. herquei* was calculated as the number of samples from which *P. herquei* was successfully isolated dividing by the total number of samples examined. Isolation rates of *P. herquei* associated with newly oviposited eggs, ovary eggs, cradle leaves, cradles and mycangia were compared by one-way ANOVA. When a significant main effect was detected, an LSD multiple comparison test was performed. All the statistical analyses were performed by SPSS 17.0 software.

Results

Behaviors and ultrastructure of adult

Only female weevils have the mycangial structure, which is located between the two rear legs, at the junction between sternal shield and hypoplax (as indicated by the dotted arrow, Fig. 1a). Large numbers of white fungal spores block the entrance of mycangium (Fig. 1b), such fungal spores were frequently observed in females at the egg-laying stage. Several holes perforated by numerous stiff pubescences were arranged around the mycangium (Fig. 1b), which may function as adhesion points for fungal spores. Inside the mycangium many grooves were found, in which fungal spores were stored (Fig. 1c).

The female weevil's trasi have many jagged teeth (as indicated by the dotted arrow, Fig. 1d), and the female weevils use their serrated trasi to cause many holes on cradle leaf, and the fungal mycelia grow up some days later (see Appendix Fig. S1 in supporting information). There are six rows of comb-like setae on the female's abdomen (as the solid arrow showed in Fig. 1a), and many fungal spores were also observed on that (Fig. 1e). We observed the female weevils used their comb-like setae presenting on their abdomen to brush the fungal spores towards those holes created by trasi. While the male weevils do not take part in fungi cultivation, their ventral setae have almost disappeared (Fig. 1f).

Isolation rates of P. herquei on different materials

Isolation rates of *P. herquei* among ovary eggs, newly oviposited eggs, cradle leaves, cradles and female mycangia were significantly different from each other ($F_{4,10} = 298.250$, $P < 0.001$). *P. herquei* was not isolated from any ovary eggs, but was successfully isolated up to 81.11% from newly oviposited eggs, and followed by 76.67% cradle leaves and 77.77% female mycangia (Table 1), respectively. However, the mean isolation rate of *P. herquei* on cradles was 87.77%, significantly higher than newly oviposited eggs ($P = 0.049$), cradle leaves ($P = 0.004$), and female mycangia ($P = 0.007$) (Table 1).

Sequence variations

A total of 60 isolates (15 from cradle leaves, cradles, eggs and mycangia, respectively) were finally sequenced, and sequences were submitted in NCBI. The length of the ITS region, which included partial 16S rRNA, ITS 1–5.8S-ITS 2, and partial 28S rRNA, ranged from 560 bp to 670 bp, and a fragment of about 580 bp was amplified and sequenced from the COI gene of all samples (Appendix Table S1).

The ITS sequences among isolates showed high identity (>99%), and pairwise genetic distances between the sequences of four hosts (newly oviposited eggs, cradle leaves, cradles and mycangia) as well as with *P. herquei* (FR670310) from GenBank were very small, ranging from 0 to 0.004 (Appendix Table S2), indicating that they belong to the same fungal species. The NJ tree based on the ITS sequences showed very small intraspecific divergences among sequences, and all of them were assembled in the same clade with *P. herquei* (FR670310), supported by 100% bootstrap values (Fig. 2a).

Concordant with the results of the ITS sequences, the similarity of the COI gene sequences among four sequences as well as with *P. herquei* (JN626061) were very high (>98%), and all pairwise genetic distances among them ranged from 0 to 0.009 (Appendix Table S1). Based on the COI sequences, the topological structure of the NJ tree was similar to that of ITS, and all the sequences formed one large group with *P. herquei* (JN626061) on the COI NJ tree. Unlike the ITS NJ

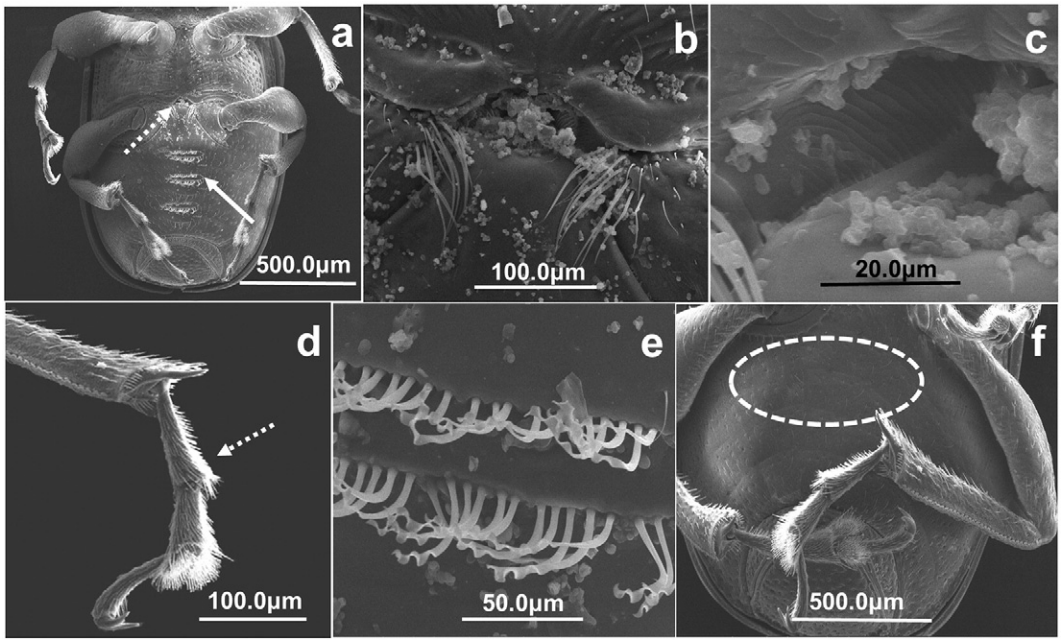

Fig. 1. Details of the specialized ultrastructures of the female adults (a–e) compared with the male adults (f). (a) The female weevils possess a mycangium (shown by the dotted arrow) and comb-like setae on their abdomen (shown by the solid arrow). Many fungal spores were found on the entrance (b) and inside of the mycangium (c). (d) Tarsi with many jagged teeth. (e) Fungal spores on the setae. (f) The setae were not present on the male's abdomen.

Table 1
Isolation rates of *P. herquei* associated with ovary eggs, newly oviposited eggs, cradle leaves, cradles and female mycangia of *E. chinensis*. Each isolation was repeated three times. Values with different letters were significantly different at the $P < 0.05$ level by LSD test.

Source	Total number of sample	Frequency of *P. herquei* (mean ± SE, %)
Ovary eggs	24	0 a
Newly oviposited eggs	30	81.11 ± 1.92 b
Cradle leaves	30	76.67 ± 3.33 b
Cradles	30	87.77 ± 1.92 c
Mycangia	90	77.77 ± 6.94 b

SE: standard error.

tree, the sequences sequenced in our study diverged from *P. herquei* (JN626061) from GenBank, and formed a subgroup with a low degree of divergence (Fig. 2b).

Discussion

Transovum transmission (on the surface of eggs) and transovarian transmission (within eggs) are the two most common modes utilized by insects to vertically transmit their microbial symbionts (Burden et al., 2002; Mann et al., 2011; Siegel et al., 2000). However, in the present study, fungal symbionts were not isolated from ovary eggs, but were successfully isolated from newly oviposited eggs. These results indicated that eggs were associated with fungi during the course of oviposition rather than during the egg formation process in the ovary. In fact, many insects were also found to inoculate fungi during oviposition process, for example, the gall midge tribe *Asphondyliini* females transmit the conidia into the oviposition channel after egg deposition (Rohfritsch, 2008), and the lizard beetle *Doubledaya bucculenta* deposits the

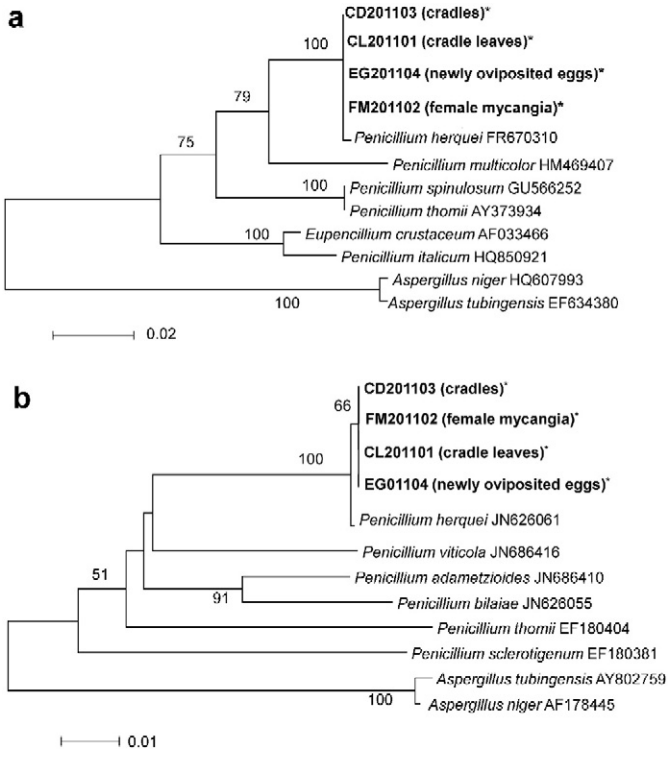

Fig. 2. Neighbor-joining trees inferred from DNA sequences of ITS regions (a) and CO1 gene sequences (b). Bootstrap values (1000 replicates) of 50% or higher are shown at the nodes. Fungal isolates examined in this study with *are discriminated by the sources of the isolates in parentheses.

symbiotic yeast on the eggs and the inner surfaces of dead host plants though an ovipositor-associated mycangium (Toki et al., 2012). Inoculating fungal spores to the oviposition places may help insects to form an intimate association with their symbionts.

Mycangial structures have been reported to play essential roles in storage and transmission of symbionts (Grebennikov and Leschen, 2010; Mueller et al., 2005; Six, 2012; Tanahashi et al., 2010). Besides in mycangium, many fungal spores adhered to the comb-like setae on the female's abdomen, indicating that these setae also play an important role in fungal transportation from mycangia to plant leaves. Similarly, both Sakurai (1985) and Wang et al. (2015) also observed the female *Euops* weevils use their comb-like setae of the abdomen to brush the fungal spores towards punctured holes. As the male weevils do not take part in fungal transportation and cradle construction activities, their ventral setae have almost disappeared (Fig. 1f) (Sakurai, 1985; Wang et al., 2015). We need to further explore the mechanisms: 1). How and when the fungal symbiont is incorporated into the mycangia, and 2). How it is selectively transmitted during the course of insect reproduction.

High frequency of *P. herquei* was isolated from cradle leaves, suggesting that fungal symbiont had been inoculated on the leaf surface before the female weevils deposited eggs under oviposition stage. Sakurai (1985) reported that a clump of hyphae with uniform appearance had covered the cradle leaf after a few days nibbled by the female adults of *E. splendidus*. Wang et al. (2015) reported that the females use their protibia and maxillary to cut the leaf and cultivate the fungal spores. Our field observations showed that after a cradle leaf was cut from the whole leaf, it was punctured multiple pairs of holes by the serrated tarsi of the female adults, several circles of traces appeared on the back of cradle leaves, and the fungal mycelia grew up after some days (Appendix Fig. S1). Therefore, the female's tarsi are also used to create places for cultivating fungi. In our study, the isolation rate of *P. herquei* on cradles was significantly higher than newly oviposited eggs, cradle leaves and mycangia. As per our close observations, some female weevils did not carry any fungal spores in their mycangia possibly they were not at oviposition stage. Sakurai (1985) and Wang et al. (2015) also reported that fungal spores could not be found in the mycangia of teneral females. It is still unknown the location and the lifecycle of the symbiotic fungus in the bodies of females that are not at oviposition stage.

Interesting, we found that the female weevils sometimes abandoned some cradle leaves during the cradle construction process. We assume that the female weevils may be able to recognize the ideal oviposition places for offspring, and they might not roll the plant leaves that are not suitable for their larvae development. Some leaf-rolling weevils have been reported to be able to recognize leaf size and select oviposition sites for their offspring (Kobayashi and Kato, 2004; Sakurai, 1990). After a cradle being constructed, it is cut down from the tree by the female adults, the moist environment and the antibiotic production ability of *P. herquei* cause the superior abundance on cradle. However, even for cradle, the isolation rate of *P. herquei* was not 100% (<90%), some fungal spores may not be grow up due to hot and dry weather of summer.

The internal transcribed spacer (ITS) and COI barcode are two useful tools for fungal species identification and phylogenetic inference (Hajibabaei et al., 2007; Nilsson et al., 2008). DNA sequence analysis of the ITS region and COI barcode results confirmed that all fungal symbionts were close relatives of *P. herquei*. However, the four isolates examined in our study were clustered into one branch in the ITS NJ tree, but were separated from the *P. herquei* (accession number was JN626061) from Gen-Bank in the COI NJ tree. Although it's considered that COI barcode is more efficient in comparing the intraspecific variations of fungi than ITS (Schoch et al., 2012; Seifert et al., 2007), especially for the *Penicillium* genus (Seifert et al., 2007), we think it is also probably due to length difference between the fragments sequenced in our study and the widely available sequences.

Several species of leaf-rolling weevils use *Penicillium* as symbionts (Kobayashi et al., 2008; Li et al., 2012; Sakurai, 1985). Previous studies suggested that the defensive role of *P. herquei* in protecting the fungal garden (cradle), Li et al. (2012) found that *P. herquei* could significantly decreased the cellulose content of the knotweed leaves, and showed great antibiotic properties against two pathogenic fungi that attack the cradle. Wang et al. (2015) reported that *P. herquei* could produce an antibiotic, (þ)-scleroderolide, to protect the cradle against potential infection. Besides of defensive role, *P. herquei* may be also included in the larvae's diets. Both Wang et al. (2010) and we have observed *E. chinensis* larvae eating fungal mycelia under a hand lens. Many social insects, such as ants, bark beetles, and ambrosia beetles are found to be fungivorous (Mueller, 2012; Mueller et al., 2005; Six, 2012), and some insects feeding on vascular plants also eat fungi (Jonsell and Nordlander, 2004). In those cases, it is difficult to determine whether fungi are used in combination with plant materials or as an independent food resource (Remén et al., 2010). Therefore, we suppose that *Penicillium* fungi may be an important nutritional resource for leaf-rolling weevil larvae, although further experiments are needed to investigate the potential contributions of *Penicillium* fungi to the larval diet.

In conclusion, our study demonstrates that the symbiotic fungus *P. herquei* is transmitted by the female *E. chinensis* before oviposition. Besides previous reported specialized structures such as maxillary palps and comb-like setae on the female bodies, we found that the female's tarsi are also used for preparing inoculation sites. This study clearly elucidates the transmission mode of the symbiotic fungi with the *Euops* weevils, and might help us to understand the evolutionary adaptation of fungiculture mutualism with other insects and host plants. In future we need to explore the mechanism in depth by using FISH, immune gold labeling and micro array to get the interactive signal transaction between the *Euops* weevils and their symbiotic fungi.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aspen.2016.06.006.

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