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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.

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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 egg 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°23'24.50.01), 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 from 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 Tiagent DNA Mini Kit (Beijing, China) and ITS region was amplified by the primers ITS 1 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.

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