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**Experimental infection of the ant *Polyrhachis furcata* with *Ophiocordyceps* reveals specificity of behavioural manipulation**

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## Abstract

Recent studies revealed that fungi of the *Ophiocordyceps unilateralis sensu lato* complex are highly host-specific. Infected ants leave their colony, wander singly with convulsions and eventually bite firmly vegetal substrates in the low vegetation and maintain this position until death. Subsequently, a fungal stroma grows from the back of the ant's head and produces spores. We experimentally infected *Polyrhachis furcata* ants by injecting them with suspensions of blastospores from three closely related fungi of the *O. unilateralis* species complex: *O. polyrhachis-furcata*, *O. camponoti-leonardi* and *O. camponoti-saundersi*, pathogens isolated from the ant species *Polyrhachis furcata*, *Camponotus leonardi* and *Camponotus saundersi*. We monitored the survival and behaviour of ants for 30 d after blastospore injection and compared the results with negative controls. Our results showed that the number of dead ants on the floor did not show significant differences across treatments. However, the typical erratic wandering behaviour and death grip display were observed only when ants were infected by their specific parasite, *O. polyrhachis-furcata*. Experimental ants initiated death grip between 13 and 17 d after infection, and stayed locked in the position. We suggest that the inability of *Ophiocordyceps* fungi to manipulate the behaviour of non-host ant species might be responsible for the observed specificity.

## Keywords

*Ophiocordyceps unilateralis sensu lato*, *Polyrhachis furcata*, host-pathogen specificity, behavioural manipulation, experimental infection

The entomopathogenic fungi of the *Ophiocordyceps unilateralis* species complex are specialized parasites of ants (Evans & Samson 1982; 1984). Production and dispersion of spores rely on modifications of the behaviour of infected ants and count among the most fascinating examples of parasite manipulation of host behaviour, popularly known as the zombie ants' syndrome (Oi & Pereira 1993; Andersen et al. 2009; Hughes et al. 2011). Infected ants singly leave their colony and climb into vegetation by displaying erratic walking and convulsions. They finally bite a vein on the backsides of leaves, the edge of a leaf or a small twig in the low vegetation and die in that position (Andersen et al. 2009; Pontoppidan et al. 2009; Hughes et al. 2011; Mongkolsamrit et al. 2012). Thereafter, a stroma emerges from the back of the ant's head and the fungus develops a fruit body and releases spores. Using experimental infection, de Bekker et al. (2014) confirmed that the death grip display of two North-American *Camponotus* species results from infection by *Ophiocordyceps*.

The *O. unilateralis* species complex is composed of many species and each one is found growing on different ant species, suggesting host-driven speciation (Evans et al. 2011; Luangsa-Ard et al. 2011; Kobmoo et al. 2012; 2015). Morphological and molecular studies of *Ophiocordyceps unilateralis* in Thailand revealed six fungal species, namely *O. polyrhachis-furcata*, *O. camponoti-saundersi*, *O. camponoti-leonardi*, *O. halabalaensis*, *O. septa* and *O. rami*, parasitizing the ant species *Polyrhachis furcata*, *Colobopsis saundersi*, *C. leonardi*, *Dinomyrmex gigas*, *Camponotus* sp.1 and *Camponotus* sp.2, respectively (Luangsa-Ard et al. 2011; Kobmoo et al. 2012; 2015). The mechanisms underlying host-parasite specificity in this system are still unclear. However, experimental injection of hyphae of a North American *Ophiocordyceps* species into host and non-host ant species showed that the fungus could induce the typical death grip display only in host species (de Bekker et al. 2014). In this study we tested the hypothesis that host specificity of *O. unilateralis* s. l. species in Thailand arises from the inability of the fungus to manipulate the behaviour of ant species other than its specific host ant species. For this, we infected *Polyrhachis furcata* ants experimentally with three fungal species of *O. unilateralis* s. l., i.e. *O. polyrhachis-furcata*, *O. camponoti-saundersi* and *O. camponoti-leonardi*, and recorded the ant behaviour.

One colony of *P. furcata* was collected in Khao Yai National Park, Thailand, and reared in the laboratory at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani, under 25 °C, 40-55 % moisture and 10 h exposure to neon light. Ants were fed every 2 d with 15 % honey agar (distilled water 85 ml; honey 15 ml; agar 1.5 g) and were provided a cotton ball saturated with water. They were kept in the lab for one month before experiments to acclimatize to laboratory conditions.

One of each of three fungal species, *O. polyrhachis-furcata* (NK 298), *O. camponoti-leonardi* (NK 257) and *O. camponoti-saundersi* (NK 270) containing mature perithecia was collected in Khao Yai National Park for NK 298 and NK 270, and in the Wiang Pa Pao district, Chiang Rai province, for NK257. Ascospore isolation was done within a few hours of collection. Each specimen was fixed to the lid of a Petri dish using petroleum jelly or tape. The lid was placed over the dish filled with Potato Dextrose Agar (PDA) to capture released ascospores on the PDA. Plates were kept in a moist chamber at 20 °C for 2-3 d. The PDA was checked every six hours for discharged ascospores. These were then transferred into Grace's insect cell medium (Gibco® lot no 1294253) (Wongsa et al 2005). The ascospores developed into blastospores in this medium by budding. A volume of 0.25 ml of this suspension was transferred into 1 ml of Grace's insect cell medium. After blastospores had grown for 3 weeks, 1 ml of suspension was transferred into 25 ml of Grace's insect cell medium and kept in an incubator at 25 °C for 1 or 2 weeks. The concentration of final stock suspensions for the three fungal species was adjusted to  $2.5 \times 10^7$  spores/ml after measurement using a hemocytometer. The infection was performed by injecting 0.5 µl of blastospore suspension using a Hamilton Gastight Syringe (syringe: Hamilton 7653-01; needle: Hamilton 7803-05) into the thorax underneath the ant's front legs using a stereomicroscope.

For each of the three focal fungal species, beside the injection of blastospores, two other treatments, injection of sterile Grace Medium and no injection, were simultaneously applied each to three replicates of ten ants (i.e. 270 ants in total). The effects of the different fungal species were tested successively at 1 week interval. Each group of ten ants was placed in a plastic box (12 cm x 17 cm x 6.8 cm) containing three artificial small twigs with three leaves each and a shelter composed of a

Petri dish covered with aluminium foil. The material was sterilized using 70% ethanol before use to avoid contamination with other fungi. Feeding and laboratory conditions were the same as described above. The numbers of living ants, ants displaying death grip and dead ants on the box floor were recorded every day for 30 d. At the end of the experiment, all dead ants were kept, both those dead on the floor and those displaying death grip, in order to monitor the fungal development from the ants' body for one month. Fungal growth out of the ant's body was not observed. Moisture was then increased as this is an important requirement for fungal growth; two cotton balls saturated with water were put in the boxes and supplied with water everyday. However, opportunistic fungi such as *Penicillium* spp. and *Aspergillus* spp. quickly covered the dead ants, precluding the use of DNA sequencing for confirming the fungal species present inside the cadavers.

Several ants were found dead on the first day of the experiment, in particular in the groups that were injected (either with Grace Medium or with an inoculum). The injection procedure, and not the inoculum, was likely to be responsible for early death. During the first 15 d, the number of dead ants on the floor was lower for the control with no injection than for the two other treatments – injection of Grace Medium and of the inoculum– and was similar for these two treatments, and for all the three *O. unilateralis* species tested (Supplementary Table 1). After 15 d of experiment, the number of dead ants on the floor tended to increase for the ants injected with the inoculum, in particular when injected with *O. camponoti-saundersi* (Fig. 1), suggesting a possible negative effect of the inoculum on the survival of the ants.

Interestingly, the only treatment that induced death grip was the infection with *O. polyrhachis-furcata*, the specific *O. unilateralis* species of *P. furcata* (Fig. 1). The death grip occurred 13 d after inoculation and increased in number of ants until 17 d after inoculation, then remained stable. This treatment also induced the typical wandering behaviour with convulsions (Fig. 1 and Supplementary material), while biting an artificial leaf, similar to the behaviour observed in the field in ants naturally infected by the *O. unilateralis* s. l. species. Such behaviour was not observed in the other treatments. Our results show that among the three entomopathogenic fungi tested, only the pathogen of *P. furcata* ants was successful in manipulating the behaviour of this ant species, although

the three fungi are closely related (Kobmoo et al. 2012). This is consistent with de Bekker et al. (2014) in which experimental infection of ant species with an *O. unilateralis* s. l. species from North America would only induce wandering walking and death grip on the fungus's specific host ant species.

Previous attempts to infect ants by spraying spores on the ants' body were unsuccessful (personal data). Our latest results, obtained for the first time in a tropical Southeast Asian region and in accordance with the evidence from North America (de Bekker et al. 2014) suggest that the inability of *Ophiocordyceps* fungi to manipulate the behaviour of non-host ant species might be responsible for the observed specificity throughout the distribution area of *O. unilateralis* group. When various host-parasite species pairs occur in syntopy, as is the case for the three focal pairs targeted in our studies (Kobmoo et al. 2012), it is likely that non-host ant species would be infected by each *Ophiocordyceps* species but would represent a dead-end for the development of the fungus. In conclusion, the specificity between *O. unilateralis* s. l. fungi and their ant hosts does not rely on the evolution of chemical defences by ants at the cuticle level but involves the mechanism underlying host behaviour manipulation by the parasite inside the ants' body. A specific match between the metabolites produced by the fungus within the ant's body and the functioning of the nervous system of the ant is certainly the key for a successful infection (de Bekker et al. 2014).

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insect pathogenic fungus *Cordyceps unilateralis*, *Mycological Research* 109, 936–940.

Figure Caption:

**Figure 1:** Number of ants dead on the floor (DF) or displaying death grip (DG) during 30 d after injection of blastospores of *Ophiocordyceps polyrhachis-furcata* (blue), *O. camponoti-saundersi* (red) or *O. camponoti-leonardi* (black). The picture shows a *Polyrhachis furcata* ant displaying death grip on an artificial leaf.

