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The attraction of *Tremex apicalis* (Hymenoptera, Siricidae, Tremecinae) and its parasitoid *Ibalia japonica* (Hymenoptera, Ibalidae) to the fungus *Cerrena unicolor*

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Abstract

Woodwasps (Hymenoptera: Siricidae) are saproxylic insects and a common forest pest. Siricid woodwasps are classified into two subfamilies: Siricinae and Tremecinae. All known symbiotic fungi of Siricinae are in the genus *Amylostereum* Boidin while some species of Tremecinae have been observed to have a relationship with the fungus *Cerrena unicolor* (Bull.) Murrill. Previous studies about the host searching behavior of woodwasps and their parasitoids have focused primarily on the subfamily Siricinae.

We analyzed the role of *C. unicolor* volatiles on the host searching behavior of *Tremex apicalis* Matsumura (Hymenoptera: Siricidae: Tremecinae) and its parasitoid *Ibalia* (*Tremibalia*) *japonica* Matsumura

(Hymenoptera: Ibaliiidae). The results of an olfactory response experiment indicated that the females of *T. apicalis* and its parasitoid find their respective hosts using volatiles from *C. unicolor*. Using DNA barcode, we identified basidiocarps on the trees infested with *T. apicalis*. The basidiocarps were all white-rot fungi that cause sapwood decay, including *C. unicolor*. Two additional species that we identified belonged to genera closely related to *C. unicolor*.

Woodwasp species are known to carry symbiotic fungi in a pair of specialized sacs called mycangia. Notably we found that mycangia-like structures were absent in the abdomens of *T. apicalis* females. To the best of our knowledge, *Xeris spectrum* (Linnaeus) (Hymenoptera: Siricidae) is the only reported example of woodwasp species that do not contain symbiotic fungi in their bodies.

Our results suggested that: (1) *T. apicalis* females search for host wood that is already infected with sapwood decaying fungus using volatile compounds; (2) *T. apicalis*' female parasitoid also uses volatile compounds from fungus to locate wood that is infested with its potential host.

Keywords

Woodwasp, horntail, host searching, mycangia, saproxylic insect, *Tremibalia*, Y-tube

Introduction

Saproxylic insects, like woodwasps in the family Siricidae and their parasitoids, locate suitable host wood/host insect-infested wood in their environment to increase their reproductive success (Feldhaar and Schauer 2018; Hilszczański 2018; Ulyshen and Šobotník 2018). Siricidae consists of two subfamilies: Siricinae and Tremecinae. Siricinae infest coniferous trees and Tremecinae infest broad-leaved trees (Morgan 1968; Schiff et al. 2012). Like most saproxylic insects, woodwasps are unable to digest structural polysaccharides such as lignocellulosic compounds because they lack the necessary cellulolytic enzymes (Fukuda and Hiji 1997; Slippers et al. 2012).

Many woodwasp species carry fungal symbionts in their mycangia. Symbiotic fungi are transferred to host wood during oviposition and hatched larvae feed on the fungus-infected wood. To date, all known symbiotic fungi of Siricinae are in the genus *Amylostereum* Boidin. A limited example of Tremecinae woodwasps demonstrates that this subfamily is associated with fungus *Cerrena unicolor* (Bull.) Murrill (Stillwell 1967; Tabata and Abe 1995; Pazoutova and Srutka 2007; Kuramitsu et al. 2016).

Siricinae woodwasps use semiochemicals emitted by trees to locate host wood. For example, Siricinae species of *Sirex*, *Urocerus* and *Xeris* (Hymenoptera: Siricidae: Siricinae) are attracted to monoterpene hydrocarbons from host pine trees (Sato and Maeto 2006; Matsumoto and Sato 2007; Coyle et al. 2012; Matsumoto and Sato 2012; Erbilgin et al. 2017; Kües et al. 2018). European woodwasp, *Sirex noctilio* Fabricius, is similarly attracted to the volatiles from its fungal symbiont, *Amylostereum areolatum* (Chaillet ex Fr.) Boidin (Fernández Ajó et al. 2015).

Like *S. noctilio*, egg-larval or larval endoparasitoids of Siricinae, *Ibalia* (*Ibalia*) spp. (Hymenoptera: Ibaliiidae) locate their hosts using the symbiotic fungi volatiles of their hosts (Madden 1968; Spradbery 1974; Martínez et al. 2006; Jofré et al. 2016; Kües et al. 2018; Table 1). For example, *Ibalia* (*I.*) *leucospoides*, a parasitoid of woodwasp *S. noctilio*, is attracted to the volatiles from their fungal symbiont (Martínez et al. 2006;

Table 1. Relationship of IbaIID parasitoids and their host.

IbaIID parasitoids	Host woodwasps	Host trees of host woodwasps	Symbiotic fungi of host woodwasps	References*
<i>Ibalia (Ibalia)</i> spp.	Siricinae	Coniferous trees	<i>Amylostereum</i> spp.	1, 2, 3, 4, 5
<i>Ibalia (Tremibalia)</i> spp.	Tremecinae	Broad-leaved trees	<i>Cerrena unicolor</i>	1, 2, 3, 4, 6, 7, 8

* 1 Nordlander and Liu (1994), 2 Choi et al. (2013), 3 Morgan (1968), 4 Schiff et al. (2012), 5 Tabata et al. (2012), 6 Stillwell (1967), 7 Pazoutova and Srutka (2007), 8 Tabata and Abe (1995).

Pietrantuono et al. 2012). Parasitoid wasps exhibit an antennal palpating and ovipositor probing response to discs of fungus impregnated agar (Spradbery 1974).

Information about the host wood/host insect searching behavior of Tremecinae and their parasitoids, *Ibalia (Tremibalia)* spp., is limited. The information available focuses on the attraction of *Tremex columba* (Linnaeus) (Hymenoptera: Siricidae: Tremecinae) to the wood volatile α/β -pinen (Coyle et al. 2012).

C. unicolor is the only known fungal symbiont of Tremecinae based on previous studies of *Tremex* spp. (Hymenoptera: Siricidae: Tremecinae) (Stillwell 1967; Tabata and Abe 1995; Pazoutova and Srutka 2007; Table 1). Basidiocarps which had the morphology of *C. unicolor* were present on wood infested by *Tremex apicalis* Matsumura (Kuramitsu et al. 2016). Whether or not *C. unicolor* is a symbiotic fungi of *T. apicalis* is not yet determined. To clarify the interaction between *T. apicalis* and *C. unicolor*: (1) we dissected the abdomens of *T. apicalis* females to isolate their mycangia, (2) analyzed the role of *C. unicolor* volatiles on *T. apicalis*' behavior, (3) identified basidiocarps on *T. apicalis* infested trees.

Also, we hypothesized that *Ibalia (T.) japonica* Matsumura, a parasitoid of *T. apicalis*, uses volatiles from *C. unicolor* to locate trees with potential host woodwasps. To test this hypothesis, we investigated the role of fungus volatiles on the host searching behavior of parasitoid *I. japonica* under laboratory conditions.

Materials and methods

Site of study and host trees

Our field survey and insect collection was conducted at Tsukuba Experimental Forest, University of Tsukuba (36°07'10"N; 140°05'50"E (DMS), ca. 25 m a.s.l.), Tsukuba, Ibaraki Prefecture, Honshu, Japan. We found four *T. apicalis* infested trees belonging to different families from 2016 to 2018 (Table 2). All *T. apicalis*, parasitoids and basidiocarps used for dissection, observation and behavioral experiments were collected from these four trees.

Observations on the abdominal organs of female *T. apicalis*

Twenty *T. apicalis* females were collected from the aforementioned trees (Table 2). *T. apicalis* females were killed using ethyl acetate. The dissection method was based on

Table 2. Host trees from which woodwasps, parasitoids and basidiocarps were collected.

Tree no.	Tree species	Diameter at breast height	Emergence of <i>T. apicalis</i> / <i>I. japonica</i>			Year of basidiocarp collection
			2016	2017	2018	
1*	<i>Swida macrophylla</i> (Wall.) (Cornales: Cornaceae)	40 cm	yes / yes	yes / no	no / no	2016
2	<i>Euptelea polyandra</i> Sieb. et Zucc. (Ranunculales: Eupteleaceae)	19 cm	no / no	yes / yes	yes / yes	2018
3	<i>Fraxinus spaethiana</i> Lingelsh (Scrophulariales: Oleaceae)	23 cm	–**	yes / yes	no / no	no fungi
4	<i>Magnolia liliiflora</i> Desr. (Magnoliales: Magnoliaceae)	44 cm	–**	yes / yes	yes / yes	2018

* This tree was the same tree that Kuramitsu et al. (2016) studied.

** These trees were not observed in 2016.

previous studies (Thomsen and Harding 2011; Li et al. 2015). The abdomen was removed using micro scissors under a stereomicroscope (Leica MZ12). The dorsal plates were removed from the abdomen. A female *Tremex longicollis* Konow, which is known to have the mycangia (Tabata and Abe 1995), was also dissected using the same method. The *T. longicollis* female was collected in Yokohama, Japan on November 12, 2017.

Extracting DNA from basidiocarps and PCR amplification of fungal ribosomal DNA

Collected basidiocarp surfaces were removed to avoid potential contamination. The samples were then ground into a fine powder using a pestle, mortar and liquid nitrogen. Fungal DNA was extracted from the powdered samples by using DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions. Each extracted DNA sample was used as a PCR template to amplify an Internal Transcribed Spacer (ITS) region by using KOD FX Neo (Toyobo) following the manufacturer's instructions. Reactions were performed with 25 µl mixture containing KOD FX Neo, 2×buffer for KOD FX Neo, 2 µM dNTP, 0.3 µM of each primer. Primers used to amplify fungal ITS region were ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAA-GTAAAAGTCGTAACAAGG-3') (White et al. 1990). PCR reactions were performed in the following order: 94 °C for 2 minutes, followed by 40 cycles of 98 °C for 10 seconds, 50 °C for 30 seconds, and 68 °C for 90 seconds. PCR products around 650 basepairs were purified using a QIAquick Gel Extraction Kit (QIAGEN).

Sequencing and molecular identification

Sequence reactions were performed with BigDye Terminator v3.1 (Thermo Fisher Scientific) followed by purification using BigDye Xterminator (Thermo Fisher Scientific). The Sanger method was applied to determine DNA sequence of ITS region using

Applied Biosystems 3130 (Gene Research Center, University of Tsukuba) and commercial sequencing services (Macrogen Japan and Eurofins Genomics). Fungi species were identified using the UNITE database (<http://unite.ut.ee>) (Kõljalg et al. 2013). ITS sequences were deposited to Genbank (accession numbers are: *Cerrena unicolor*, MH645754; *Daedaleopsis confragosa*, MH645755; *Trametes hirsuta*, MH645756).

Fungal culture disc preparation

Potato Dextrose Agar (PDA) medium (Nissui) was prepared by following the manufacturer's protocol. *C. unicolor* was obtained from the Genebank Project (National Agricultural Research Organization). For behavioral experiments, fungal cultures were inoculated with a PDA medium in a 9-cm petri dish for two weeks at 25 °C.

Olfactory responses of *T. apicalis* and its parasitoid *I. japonica*

To obtain newly emerged *T. apicalis* and *I. japonica*, we cut down woodwasp infested *E. polyandra* (Table 2, tree no. 2) and *M. liliiflora* (Table 2, tree no. 4) on November 11 and October 4, 2017, respectively. The wood was stored outside until May 2018. Newly emerged *T. apicalis* and *I. japonica* were collected upon emergence. Live adults of each species was stored in plastic containers (16 × 28 × 17 cm). All insects were allowed to mate and feed on a solution of sugar and water (30% w/w) for 24–48 hours before behavioral experiments. The olfactory preference of *T. apicalis* and *I. japonica* was examined with a Y-tube bioassay (Fig. 1a) in the laboratory (25 °C ± 1.1 °C). Arms of a glass Y-tube olfactometer (common arm 20 cm, arms 20 cm, diameter 3 cm) were connected to glass jars (17 cm × 12.5 cm) respectively. Each glass jar was connected to an activated charcoal filter. The Y-tube olfactometer was connected to an electric vacuum pump (KNF, Germany) through a flow meter. The arm side of the Y-tube olfactometer was inclined upward at 5-degrees using a metal stand (Fig. 1b). The airflow from outside passed through the charcoal, the glass jar and into the olfactometer. The flow rate of the pump was set at 1.5 L/min.

A single male or female woodwasp or parasitoid was introduced into the starting point of the Y-tube olfactometer. Its behavior was observed for maximum of 15 minutes. We recorded the choice of the wasp if it reached the finish line. If it did not make a choice within 15 minutes, the trial was recorded as, “no choice.” A total 43 *T. apicalis* (20 females and 23 males) and 57 *I. japonica* (25 females and 32 males) were tested. To avoid bias in the experimental setup, the positions of the two odors sources were exchanged after testing five woodwasps or parasitoids. Odor sources were renewed after testing five woodwasps or parasitoids. Using a binomial test, we determined the preference of both *T. apicalis* and *I. japonica* between volatiles from fungal disks and control disks.

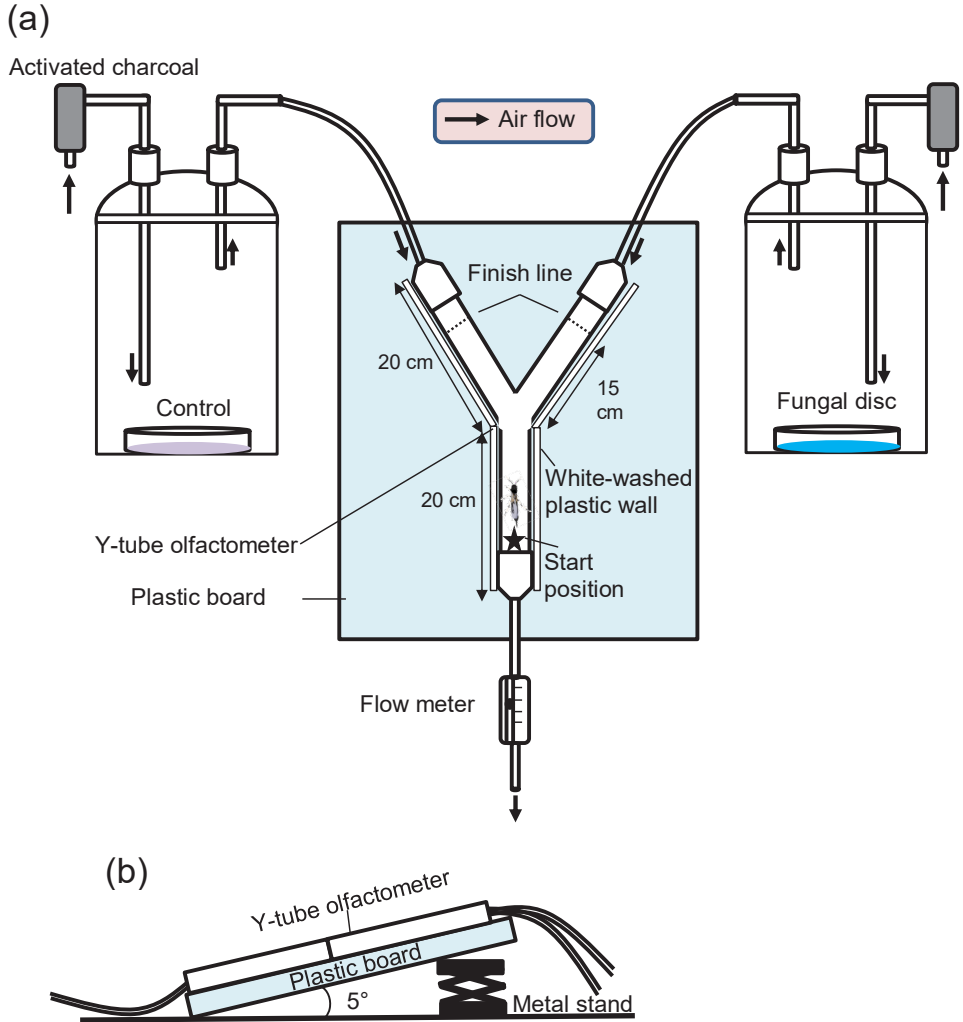


Figure 1. Set-up of the Y-tube olfactometer used to test *T. apicalis* and *I. japonica* attraction to volatiles from a fungal disc of *C. unicolor* in a top view (a) and in a side view (b) of the Y-tube olfactometer.

Results

Female *T. apicalis* abdominal organs

Abdomens of the female *T. apicalis* had ovaries that contained on average 96 eggs (Fig. 2a). A pair of mucus glands, which looked like large whitish sacs, was highly developed (Fig. 2a). A single mucus reservoir connected to the basement of the ovipositor was detected under the mucus glands (Fig 2b). The reservoir contained a sticky transparent fluid.

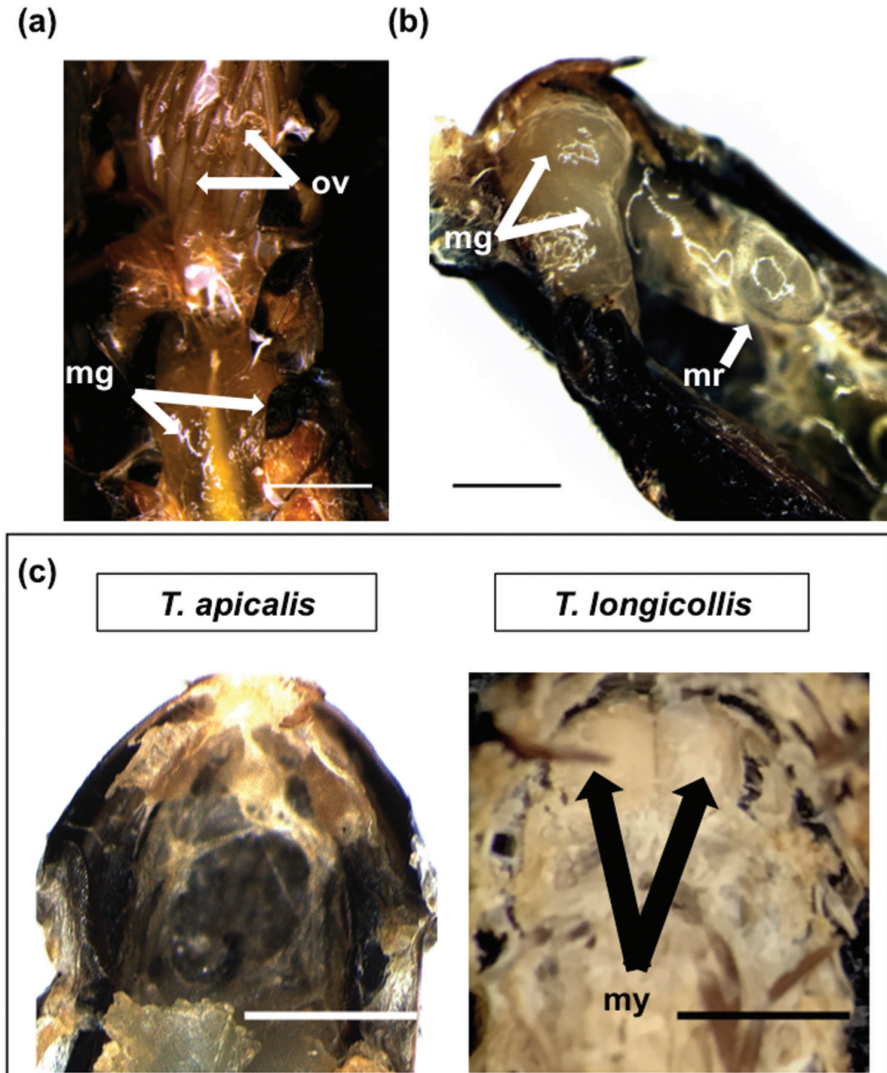


Figure 2. Abdominal organs of female woodwasps. **a** Ventral view of the dissected abdomen of *T. apicalis*. Typical internal organs, ovaries and mucus glands were easily detected. **b** Upper left diagonal view of the dissected abdomen of *T. apicalis*. A mucus reservoir was detected under the mucus glands. **c** Comparative view of dissected region of abdomens close to the ovipositor. In *T. longicollis*, the mycangia are recognized as greyish balls located close to the basement of ovipositor. There were no such sac-like structures in *T. apicalis*. Abbreviations; my: mycangia, mr: mucus reservoir, mg: mucus gland, ov: ovary. Scale bar: 2 mm.

We could not locate mycangia-like structures in the abdomen of the female *T. apicalis* while other anatomical features were nearly identical to other woodwasps. There were no visible sac-like structures located behind the base of the ovipositor where *T. longicollis* has clearly identifiable mycangia (Fig. 2c).

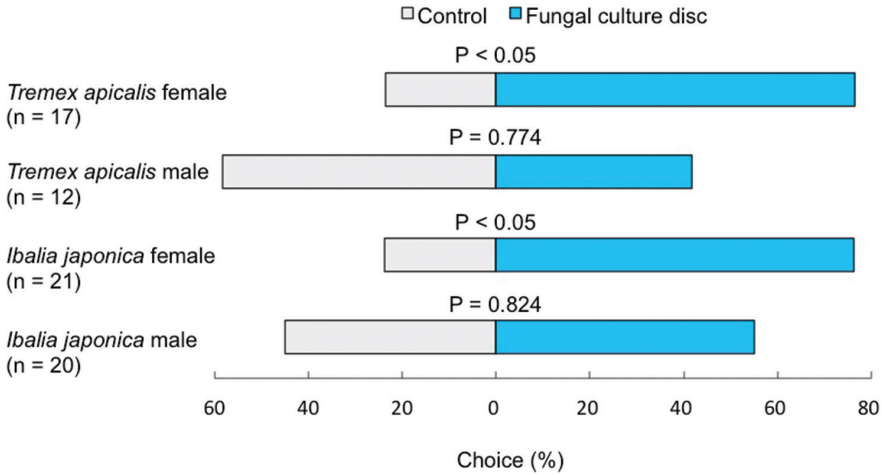


Figure 3. Percent choice by *T. apicalis* and *I. japonica* in arms of Y-tube olfactometer with the volatiles from PDA discs (control) vs. fungal culture discs.

Identification of basidiocarps on woodwasp infested trees

We performed DNA barcoding for basidiocarps found on *T. apicalis* infested trees using ITS region. The results showed that the ITS region from basidiocarps on: (1) *S. macrophylla* had a 99.67% match with *C. unicolor*; (2) *E. polyandra* had a 99.81% match to *Daedaleopsis confragosa* (Bolton) J.Schröt.; and (3) *M. liliiflora* was identical to *Trametes hirsuta* (Wulfen) Lloyd. All the fungus species we identified belong to the family Polyporaceae.

Olfactory responses of *T. apicalis* and its parasitoid *I. japonica*

In the Y-tube bioassay, 85.0% (n = 20) of *T. apicalis* females, 52.2% (n = 23) of its males, 84.0% (n = 25) of *I. japonica* females and 62.5 % (n = 32) of males chose between the volatiles from fungal and control disks. Females of *T. apicalis* woodwasps (76.5 %, n = 17, $P < 0.05$) and *I. japonica* (76.2%, n = 21, $P < 0.05$) preferred volatiles from the fungal disk to the control disks (Fig. 3). In contrast, males of both *T. apicalis* (41.7%, n = 12, $P = 0.77$) and *I. japonica* (55.0%, n = 20, $P = 0.82$) did not display a statistically significant preference for either fungal or control disks.

Discussion

T. apicalis females without mycangia feed opportunistically on rotten wood

Subfamily Siricinae has a close relationship with the genus *Amylostereum* (Schiff et al. 2012; Tabata et al. 2012). For subfamily Tremecinae, *C. unicolor* is only known

symbiotic fungus for many *Tremex* woodwasps (Stillwell 1967; Tabata and Abe 1995; Pazoutova and Srutka 2007). Basidiocarps on *T. apicalis* infested large-leaf dogwood tree (*Swida macrophylla*) were *C. unicolor*. Notably, we could not identify any apparent mycangia-like structures inside the female *T. apicalis* (Fig. 2).

Basidiocarps on other *T. apicalis* infested trees were members of the family Polyporaceae, inclusive of *C. unicolor*. These species are white-rot fungi that cause sap wood decay (Enebak and Blanchette 1989; Stajić et al. 2017; Račko et al. 2018). Our observations suggest that *T. apicalis*, which lacks identifiable mycangia, inhabits host wood that is already infected by wood decaying fungi.

Relationship between *C. unicolor* and *T. apicalis*

The female *T. apicalis*' preference for *C. unicolor* suggests that it uses volatiles from the fungus to locate suitable host wood. This strategy would be similar to female *Xeris spectrum* (Hymenoptera: Siricidae), whose mycangia is also absent and who use the odor from fungi *Amylostereum* to locate host wood (Fukuda and Hijii 1997; Matsumoto and Sato 2012).

While mycangia carrying woodwasps *Sirex nitobei* Matsumura and *Urocerus japonicus* Smith form specific relationships with a particular *Amylostereum* fungus, mycangia-less *X. spectrum* can utilize more than one particular species. For example, the *Amylostereum* fungi species has a well-documented relationship with *Sirex nitobei* and *Urocerus japonicus* respectively (Fukuda and Hijii 1997).

It is possible that *T. apicalis* employs similar strategies that take advantage of their lack of mycangia. This may provide woodwasps without mycangia with an evolutionary advantage that increases their overall chances for reproduction and survival.

Parasitoid strategies

The Siricinae parasitoid *I. leucospoides* locates its host using volatile cues from symbiotic fungi (Martínez et al. 2006; Pietrantuono et al. 2012). In our study, we demonstrated that *I. japonica* is attracted to volatile compounds from *C. unicolor* even though this fungus is not a symbiont carried in *T. apicalis*. In contrast, *I. japonica*'s potential host, *T. longicollis*, has symbiotic relationship with *C. unicolor* (Tabata and Abe 1995; Watanabe et al. 2018). In order to locate potential hosts, *Ibalia* parasitoids seem to exploit olfactory cues not only from symbiotic fungi but also fungi species that live outside of the host.

To the best of our knowledge, this is the first report that connects white rot wood decaying fungus, Tremecinae woodwasp species and its parasitoid via volatile compounds.

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