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Molecular ecological studies on the chemical disguise strategy in the socially parasitic spiny ant *Polyrhachis lamellidens*

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Abstract

Although most ant species independently establish their colonies, some socially parasitic ants build the foundation of their colonies by invading other ant (host) colonies and utilizing their workforce. When invading the host colony, many socially parasitic ants disguise their cuticular hydrocarbon (CHC) profile, also known as the chemical cue for nestmate discrimination. The strategy of chemical disguise is widespread in socially parasitic ants; therefore, elucidating the mechanism of chemical disguise will promote knowledge about the evolutionary background of social parasitism. This study investigated the ecological information and mechanism of chemical disguise in temporary socially parasitic spiny ants (*Polyrhachis lamellidens*) via field research, measuring the CHC profile of ants, performing a tracing assay with labeled substances, and analyzing gene expression levels. First, the interactions between *P. lamellidens* and their associated organisms (myrmecophiles and hosts) were elucidated. It is revealed that *Microdon katsurai* utilizes *P. lamellidens* as its host during larval and pupal stages (Chapter 2), while *P. lamellidens* is a socially parasitic ant that host *Camponotus obscuripes* (Chapter 3). These knowledges suggest that *P. lamellidens* has two faces as a host and a parasite. Then, the chemical disguise mechanisms of *P. lamellidens* were investigated based on the findings of Chapters 2 and 3. After the characteristic rubbing behavior (e.g., straddling the host worker and rubbing its body with its legs) was observed against the host workers, the CHC profile of *P. lamellidens* shifted to pronounced peaks that closely resembled those of the host workers. A reduction was observed in the aggressive behavior of the host ants against *P. lamellidens* after the rubbing behavior was performed. In addition, *P. lamellidens* acquired artificially applied labeling substances from host workers through their rubbing behavior. In contrast, gene expression profiling showed that CHC synthesis-related gene expression did not change during this behavior (Chapter 4). These results suggest that *P. lamellidens* directly obtains host CHCs and these host CHCs enable *P. lamellidens* to remain disguised during colony invasion. I believe that this study succeeded in elucidating the basic ecological information of *P. lamellidens* and some of the chemical disguise mechanisms of socially parasitic ants.

Keywords:

Temporary social parasite, *Polyrhachis lamellidens*, Cuticular hydrocarbon, Chemical disguise, Ant

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社会寄生種トゲアリ（*Polyrhachis lamellidens*）の化学偽装戦略

に関する分子生態学研究

要旨

多くのアリ種は独力でコロニーを創設するが，社会寄生アリの中には他アリ種（宿主）のコロニーに侵入し，その労働力を利用することでコロニー創設を行う種が存在する．社会寄生アリの多くは宿主コロニーに侵入する際，巣仲間識別のための化学的合図として知られる体表炭化水素（CHC）のプロファイルを偽装する．このような化学偽装戦略は社会寄生アリの様々な種で広く確認されており，本戦略のメカニズムを解明することは社会寄生の進化背景に関する知見を獲得することに繋がる．本研究では，一時的社会寄生種であるトゲアリ（*Polyrhachis lamellidens*）を対象に，野外調査，CHC プロファイルの測定，標識物質による追跡試験，遺伝子発現量解析を実施し，本種の生態情報と化学偽装メカニズムについて調査した．全てに先立ち，トゲアリとその関連生物（好蟻性生物と宿主）の相互作用を解明した．ケンランアリスアブ（*Microdon katsurai*）は幼生期にトゲアリを宿主として利用する一方で（第二章），そのトゲアリはムネアカオオアリ（*Camponotus obscuripes*）を宿主とする社会寄生種であることが判明した（第三章）．これらの知見はトゲアリが宿主と寄生者という相反する二つの側面を持つことを示す．次に，第二，第三章の知見に基づいて，トゲアリの化学偽装機構を調査した．トゲアリの CHC プロファイルは宿主働きアリに対する馬乗り行動（宿主働きアリに跨り，自身の脚で擦り付けるなどといった行動）が観察された後，宿主働きアリと類似したピークに顕著にシフトすることを確認した．また馬乗り行動を行った後，宿主働きアリによるトゲアリに対する攻撃行動が減少することも観察された．さらに，トゲアリは馬乗り行動を通して，宿主働きアリに人工的に塗布された標識物質を獲得する一方，本行動中に CHC 合成関連遺伝子の発現量は変動しないことを遺伝子発現プロファイリングにより示した（第四章）．これらの結果から，トゲアリは宿主の CHC を直接奪取し，この宿主由来の CHC によってコロニー侵入時における偽装を実現することが示唆された．本研究により，トゲアリの基礎的な生態情報と社会寄生アリの化学偽装メカニズムの一端を解明することに成功したと考える．

キーワード：

一時的社会寄生，トゲアリ，体表炭化水素，化学偽装，アリ

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List of Abbreviations

BR	Broad range
C. obs	*C. obscuripes*
$C_{22}H_{46}$	n-docosane
$C_{30}D_{62}$	n-triacontane-d62
$C_{30}H_{62}$	n-triacontane
$C_{32}D_{66}$	n-dotriacontane-d62
$C_{32}H_{66}$	n-dotriacontane
$CaCl_2$	Calcium chloride
CHC	Cuticular hydrocarbon
COI/II	Cytochrome oxidase subunits I and II
DEG	Differentially expressed gene
DIG	Digoxigenin
ECL	Equivalent chain length
FDR	False discovery rate
GC-MS	Gas chromatography-mass spectrometry
HS	High sensitivity
KCl	Potassium chloride
LC-MS	Liquid chromatography-mass spectrometry
Lys-C	Lysyl endopeptidase
$MgCl_2$	Magnesium dichloride
MS	Mass spectrometry
NaCl	Sodium chloride

NBT/BCIP	Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate
P. lam	*P. lamellidens*
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PPG	Postpharyngeal gland
PTw	Tween 20 in PBS
RT-qPCR	Reverse transcription-quantitative PCR
SSC	Saline sodium citrate
TE	Tris-EDTA (ethylenediaminetetraacetic acid)
TES	Tris-EDTA-SDS (sodium dodecyl sulfate)
TPM	Transcripts per million
Tris-HCl	Tris hydrochloride acid
tRNA	Transfer RNA
VU	Vulnerable
as	A pair of anterior spiracles
bp	Base pair
cDNA	Complementary DNA
ds cDNA	Double-stranded complementary DNA
mcr	Median carina
mRNA	Messenger RNA
ppo	Protruded pores
prp	Posterior respiratory process

Chapter 1

Introduction

1-1. Socially parasitic ant

Ants are one of the most prosperous groups of insects because they have a social structure consisting of several individuals and an advanced division of labor (Hölldobler & Wilson, 1990). Eusocial insects have evolved altruistic behavior to increase inclusive fitness; they raise broods produced by other individuals. Furthermore, in eusocial insects, two or more generations coexist in a colony, and an infertile caste exists. Termites, wasps, and ants are the commonly known eusocial insects. A common ant colony is constituted by a queen and several workers (Hölldobler & Wilson, 1990). New queens (female colony founders) and males fly away from the nest at a specific period and mate, a behavior known as the nuptial flight. At this point, the new queen obtains sperms enough for the entire lifetime and begins nest building, oviposition, and taking care of its broods on its own until the birth of the first worker. When several workers are born, the new queen concentrates only on oviposition. The worker ants conduct all other labors, such as brood raising, nest expansion, and foraging. Generally, workers are female individuals, and although most female workers have ovaries, they do not lay eggs as long as the queen exists in the colony (Endler et al., 2004).

Generally, ants perform colony foundation and maintenance without relying on other ant species. However, socially parasitic ants depend on other ant species during a part of or throughout their lives (Buschinger, 2009) (Figure 1-1). Their parasitic strategies have four main categories: xenobiosis, temporary social parasitism, dulosis, and inquilinism. The damage to the host varies according to each strategy. In the xenobiosis strategy, parasites live in the host nest without utilizing the host's workforce. This strategy minorly damages the host because it does not use the host's labor or kill it. *Polyrhachis lama* and *Polyrhachis loweryi* are socially parasitic ants (xenobiosis) (Maschwitz et al., 2000, 2003) that utilize the nest of their host ant while maintaining their workforce. Temporary social parasitism involves not only the temporary use of host nest but also its labor force in the early stages of colony foundation. In this strategy, the newly mated queen of the parasitic ants kills the host queen (e.g., *Lasius meridionalis*) (Japanese Ant Database Group, 2003). Dulosis (slave-making) differs from temporary social parasitism because it permanently uses the host's labor (host worker). The workers of slave-making ants lack work capacity. Therefore, they periodically invade the host nest and abduct worker cocoons to sustain the workforce required to maintain the colony (e.g., *Polyergus samurai*) (Hasegawa & Yamaguchi, 1994; Tsuneoka, 2008). In the inquilinism strategy,

the parasites lack the worker caste responsible for labor and depend on the host worker for all labor, such as personal care and brood raising. This parasitism is an ultimate host-dependent strategy because the worker caste necessary for labor that eusocial insects are supposed to have has disappeared (e.g., *Vollenhovia nipponica*) (Kinomura & Yamauchi, 1992).

Social parasitism has often evolved in various ant taxa (Hölldobler & Wilson, 1990; Buschinger, 2009). However, questions remain about why and how social parasitism evolved in ants. An approach to understanding the significance of the evolutionary background of social parasitism is focusing on specific strategies commonly conserved among various socially parasitic ants. These parasitic strategies have a common feature: regardless of the degree of damage inflicted on the host, the parasite will somehow deceive the host's nestmate discrimination. This phenomenon is absent in other general ant species and is a unique strategy of socially parasitic ants.

1-2. Chemical disguise

Animals perform various interindividual interactions, such as mimicry to deceive the discrimination ability of animals. Mimicry has three primary methods: visual mimicry, acoustic mimicry, and mimicking of semiochemicals. Several socially parasitic ants perform mimicking of semiochemicals to deceive the host. In addition, many myrmecophiles (solitary organisms that depend on ants for a part of or throughout their lives) perform this strategy. Several hydrocarbons exist on the ants' cuticles, functioning as waxes to protect against dryness and semiochemicals to discriminate the nestmates (Detrain et al., 1999; Howard & Blomquist, 2005; Sturgis & Gordon, 2012). The cuticular hydrocarbon (CHC) profile of ants varies among different colonies. Ants discriminate between nestmates and foreign enemies (non-nestmates) by recognizing the differences in the CHC profiles; they behave aggressively toward individuals with different CHC profiles even if nonnestmates are of the same species. Socially parasitic ants take various measures to avoid the host's attacks. For example, some strategies include not having much original CHC (chemical insignificance) so that the host does not recognize it or disturbing the host with propaganda substances. Notably, to avoid being exclusively discriminated as nonnestmates by host ants, many socially parasitic ants disguise their own CHC profile to obtain a profile similar to that of the host colony (Akino, 2008; Dettner & Liepert, 1994; Lenoir et al., 2001). Many socially parasitic ants perform this

strategy of chemical disguise (Hojo et al., 2009; Nehring et al., 2016) regardless of the detailed strategies of parasitism and the species differences.

Chemical camouflage and chemical mimicry are two chemical disguise methods (Akino, 2008; Dettner & Liepert, 1994; Lenoir et al., 2001) (Figure 1-2). Howards stated that "chemical camouflage" acquires CHCs from the host directly and "chemical mimicry" biosynthesizes the host-like CHCs (Howard et al., 1990a & b). The chemical disguise mechanism was estimated in socially parasitic ants through ecological observations (Bauer et al., 2010; Lenoir et al., 1997). However, there is little information on the quantitative level of CHCs and intricacies of chemical disguise. To understand how a particular behavior has evolutionally adapted to the habitat, the role and function of the behavior (ultimate factor) and its mechanisms (proximate factor) must be understood. The elucidation of the proximate factors allows us to discuss why the behavior mechanism was selected in natural selection. In the case of chemical disguise in socially parasitic ants, the ultimate factor is the deception of the nestmate discrimination of host ants. However, the proximate factor is the chemical camouflage and chemical mimicry. It will be possible to discuss why the mechanism was selected in natural selection only by understanding the proximate factor of chemical disguise.

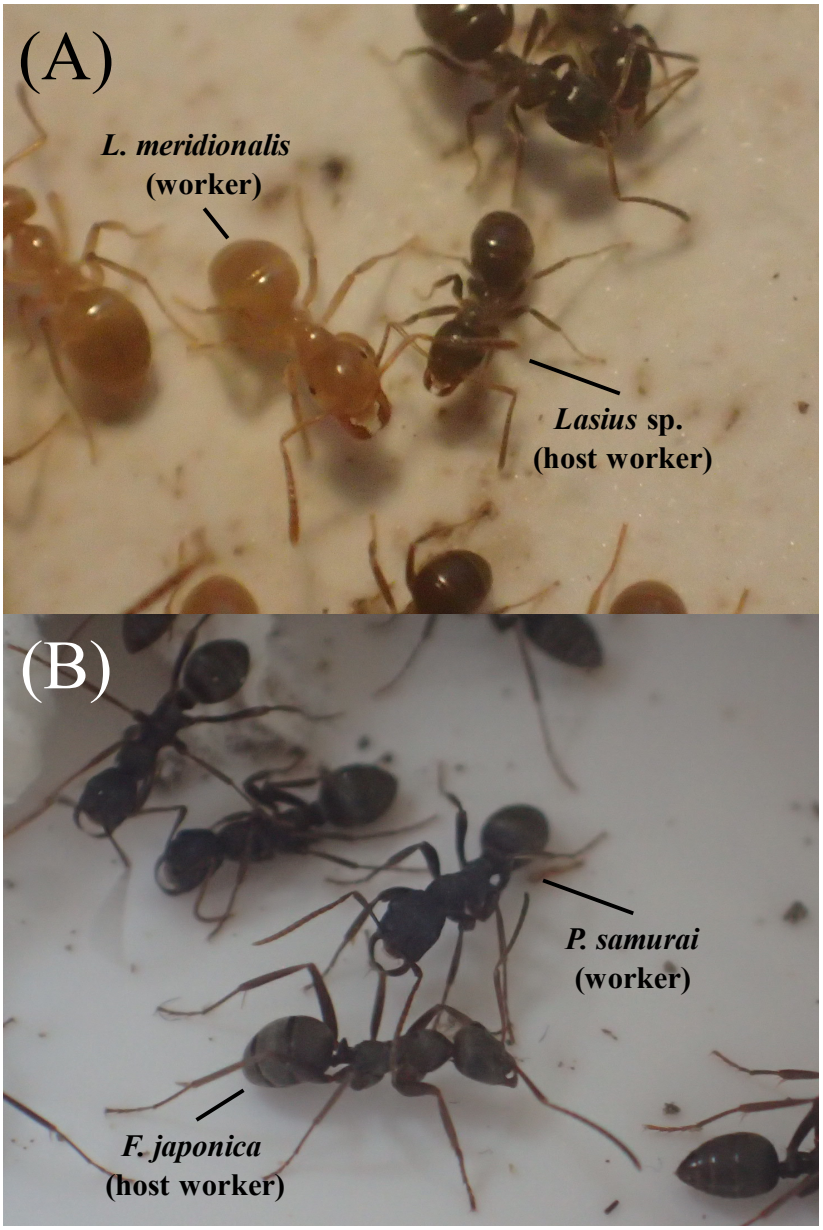


Figure 1-1. Examples of socially parasitic ants. (A) *L. meridionalis* (temporary social parasitism) and its host (*Lasius* sp.). (B) *P. samurai* (dulosis) and its host (*Formica japonica*).

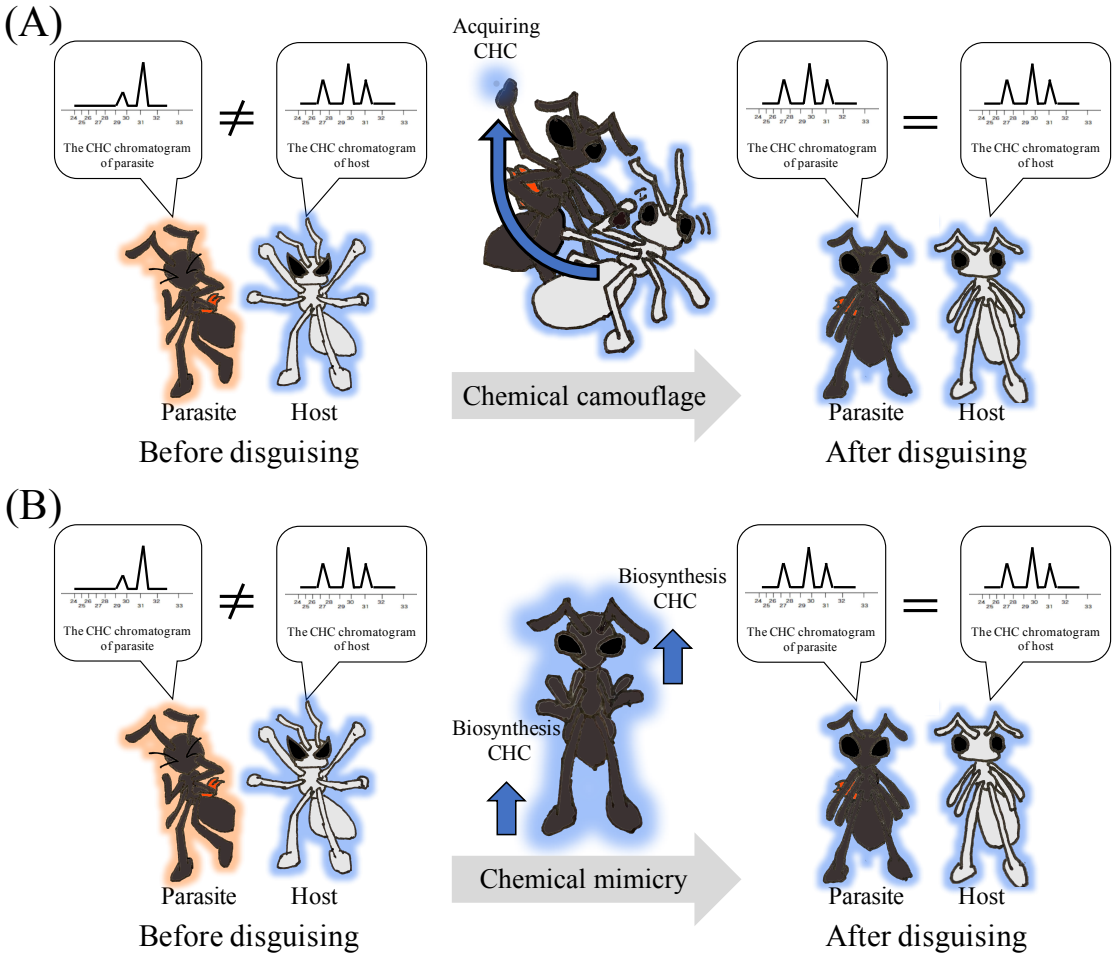


Figure 1-2. Chemical disguise methods. (A) Chemical camouflage methods. Parasites directly acquire the host CHC. (B) Chemical mimicry methods. Parasites newly biosynthesize host-like CHC profile.

1-3. Socially parasitic spiny ant *Polyrhachis lamellidens*

There are three conditions for species suitable for investigating the mechanism of chemical disguise: (1) the species can grasp the state before and after disguise; (2) it performs specialized behavior for chemical disguise; and (3) it can reproduce chemical disguise in the laboratory. First, comparative verification of these states cannot be realized unless the states before and after chemical disguise are known. To this end, the species performing a chemical disguise-specific behavior, which is a determinant of disguise, can be a suitable model. Furthermore, reproducing such disguise behavior in the laboratory enables the investigation of the disguise mechanism for the first time. This condition also includes the need for the host ants to be relatively readily available (e.g., host worker).

Polyrhachis lamellidens is a suitable candidate for this investigation, which is considered a temporary socially parasitic ant that mainly uses *Camponotus japonicus* as a host. The host range of this species extends to *Camponotus obscuripes* and *Camponotus kiusiuensis* (Japanese Ant Database Group, 2003; Kohriba, 1963 & 1966; Kubota, 1974; Kurihara et al., 2022; Sakai, 1990, 1996, & 2000; Yano, 1911) (Figure 1-3). The social parasitism of *P. lamellidens* begins with the newly mated queen invading the host nest and killing the host queen (Japanese Ant Database Group, 2003; Sakai, 1996 & 2000). In this species, the newly mated queen performs distinct behaviors when invading the host colony, including straddling the host worker and rubbing its body with its legs (Japanese Ant Database Group, 2003; Kohriba, 1963; Kubota, 1974; Kurihara et al., 2022; Sakai, 1990 & 2000). This "rubbing behavior" is unique to the newly mated *P. lamellidens* queen and is considered a chemical disguise strategy to prevent attacks by the host (Kohriba, 1963). After successful invasion, the new queen starts oviposition, and the host workers raise all eggs. As a result, all the new larvae are only *P. lamellidens*, and the host workers will continue to work for *P. lamellidens* until their death. Eventually, the colony will comprise only *P. lamellidens*. Unlike obligatory social parasitic ants (dulosis), *P. lamellidens* workers perform labor. Therefore, they can maintain colonies on their own after the host disappears. In addition, the hoverfly *Microdon katsurai* uses *P. lamellidens* as a host (Maruyama & Hironaga, 2004). If *M. katsurai* uses the socially parasitic ant (*P. lamellidens*) as the host, this relationship forms a hyperparasitism.

In summary, *P. lamellidens* is an appropriate species to use when investigating the detailed mechanisms of chemical disguise because the rubbing behavior is a distinct

chemical disguise behavior and the host of *P. lamellidens* belongs to a different genus of this species.

1-4. Structure of this dissertation

In this dissertation, the following research was conducted to reveal the chemical disguise mechanisms of *P. lamellidens*. First, in Chapters 2 and 3, the interactions between the *P. lamellidens* and their associated organisms (myrmecophiles and hosts) have been discussed. In Chapter 2, the composition of the *P. lamellidens* nest (non-mixed colony) and the symbiotic relationship with *M. katsurai* was investigated using field research and performing rearing experiments (Iwai et al., 2016). In Chapter 3, to verify the host range of *P. lamellidens*, field research, behavioral tests, and rearing experiments were performed (Iwai et al., 2021). Based on the findings of Chapters 2 and 3, the chemical disguise mechanism of a socially parasitic ant was elucidated in Chapter 4 for the newly mated *P. lamellidens* queen. In Chapter 4, the ecological role of the rubbing behavior in *P. lamellidens*-induced chemical disguise was quantitatively evaluated by measuring CHC profiles and conducting behavioral tests. In addition, the disguise strategy of *P. lamellidens* was elucidated by performing tracing assays with labeled substances and analyzing the expression of related genes (Iwai et al., 2022). The evolutionary background of socially parasitic ants has been discussed in Chapter 5 using these studies.

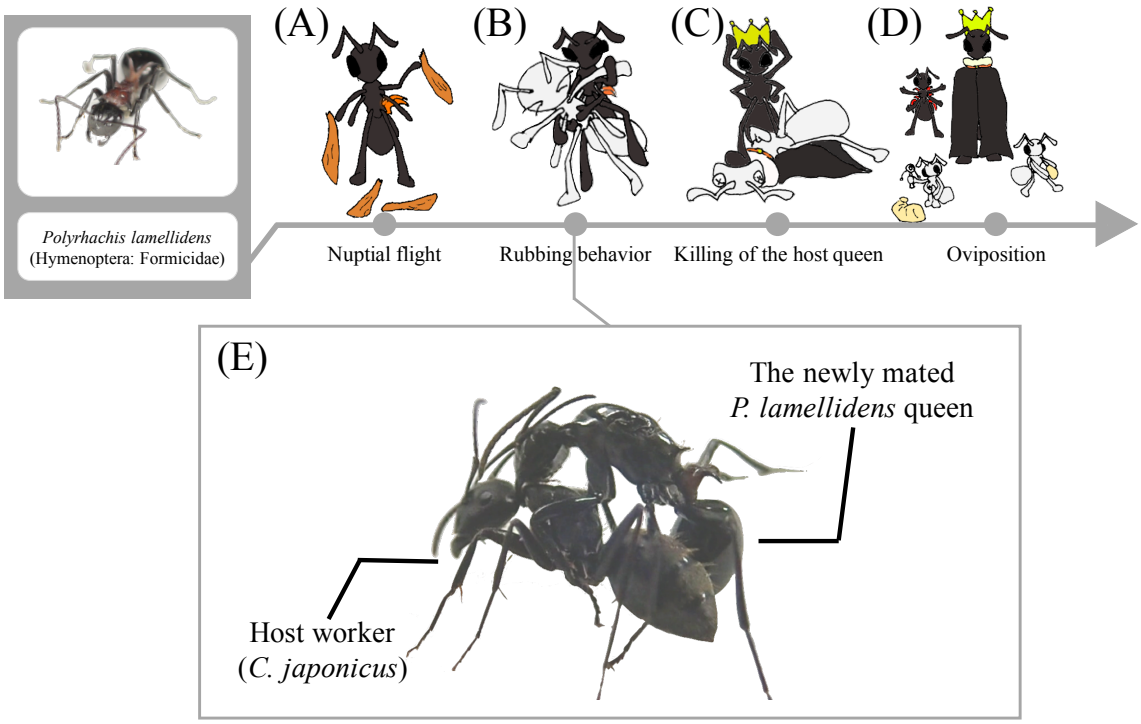


Figure 1-3. Social parasitism cycle of *P. lamellidens*. (A) The newly mated *P. lamellidens* queen searches the nest of the host ant. (B) The newly mated *P. lamellidens* queen performs the rubbing behavior against the host worker. (C) The newly mated *P. lamellidens* queen kills the host queen by biting and cutting off its neck. (D) Eggs laid by the newly mated *P. lamellidens* queen in the host colony. Host workers take care of *P. lamellidens* brood. (E) The rubbing behavior. The newly mated *P. lamellidens* queen performs the rubbing behavior against the *C. japonicus* worker.

Chapter 2

Rearing and observation of immature stages of the hoverfly *Microdon katsurai* (Diptera, Syrphidae)

2-1 Introduction

It is well known that some species of Microdontinae (Diptera, Syrphidae) spend their larval and pupal periods in ant nests. Several papers reported that larvae of some microdontine species prey on ant eggs, larvae, and pupae (Duffield, 1981; Garnett et al., 1985; Pérez-Lachaud et al., 2014; Reemer, 2013; Van Pelt & Van Pelt, 1972). At present, 454 microdontine species have been recorded (Reemer & Stahls, 2013), and the host species for 49 of them have been confirmed as myrmecophilous (Maruyama et al., 2013; Reemer, 2013). However, there are few studies on the biology of myrmecophilous microdontine species in Asia (Maruyama & Hironaga, 2004).

The microdontine species *M. katsurai* was originally described in Japan (Maruyama & Hironaga, 2004). Adult *M. katsurai* has a remarkable greenish-yellow luster. Adult stage *M. katsurai* are found near nests of the ant *P. lamellidens* Smith 1874 (Hymenoptera, Formicidae) from the end of May to the beginning of July (Hironaga et al., 1998; Katsura, 1998; Maruyama & Hironaga, 2004). Furthermore, adult females of *M. katsurai* show ovipositional behavior at the entrance of *P. lamellidens* nests (Hironaga et al., 1998; Maruyama & Hironaga, 2004). These studies imply that *P. lamellidens* is a host of *M. katsurai*. However, *M. katsurai* has never been found inside a *P. lamellidens* nest, and no interactions between these two species have been observed.

In the present study, to elucidate whether *M. katsurai* utilizes *P. lamellidens*, I tried to collect larvae of *M. katsurai* from a *P. lamellidens* nest and rear them until they developed into adults.

2-2 Materials and Methods

2-2-1 Collecting samples

I conducted field research on 5, 6, 15, and 16 March 2015 in Hosaka Natural Park (N 35°43′55″, E 138°29′18″, approximately 600 m in altitude), in the city of Nirasaki, Yamanashi Prefecture, Japan. I found a *P. lamellidens* nest in several rotten *Pinus densiflora* Siebold et Zuccarini 1842 trees that had fallen and were piled up (Figure 2-1). Each tree was approximately 100 cm in length and 20 to 50 cm in diameter.

Three larvae and four puparia of a microdontine were found in the *P. lamellidens* nest (Figures 2-2, 2-3, 2-4, and 2-5). These larvae were found on a piece of bark in the underground nest of *P. lamellidens*. Two of the puparia were attached to a piece of the bark as well, and the other two were found in wood flakes in the *P. lamellidens* nest. The

larvae with the pieces of bark were taken and transferred into a transparent plastic container in which wood flakes derived from the *P. lamellidens* nest were placed on the bottom. All workers and queens of *P. lamellidens* found in the nest were collected and transferred into a plastic container. All the collected samples were brought to the laboratory. The *P. lamellidens* colony approximately consisted of three queens, more than 1,000 workers and larvae.

Figure 2-1. The nest of *P. lamellidens*. Microdontine larvae and puparia were found in the nest of *P. lamellidens*.

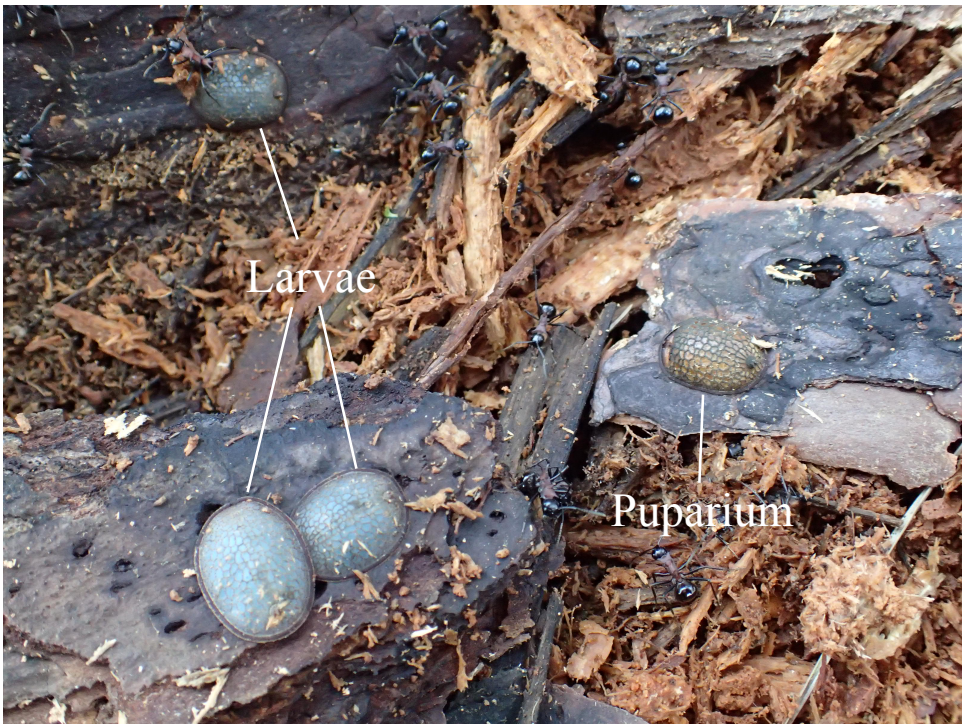


Figure 2-2. The microdontine larvae and puparium discovered in the *P. lamellidens* nest. Three larvae and one puparium were attached to pieces of bark.

Figure 2-3. A posterior respiratory process ("prp") was observed on a *M. katsurai* larva. The body size of each larva is approximately 10.0 mm.

Figure 2-4. A *P. lamellidens* worker passing by a *M. katsurai* larva. *P. lamellidens* worker is refered to as "*P. lam*".

Figure 2-5. Puparium of *M. katsurai* with a *P. lamellidens* worker. *P. lamellidens* worker is refered to as "*P. lam*".

2-2-2 Rearing animals

Due to the difficulty of identifying microdontine species by using a larva or pupa, I reared the larvae collected until adults emerged under laboratory conditions. After collecting these larvae, they were reared in the transparent plastic container used for collecting the larval samples for the first two days. Thereafter, the larvae were numbered 1 to 3 and reared individually in a new plastic container (130 mm in diameter and 59 mm in height), and wood flakes taken from the *P. lamellidens* nest were placed on the bottom. Small holes were made in the lid of each container for ventilation. two days after the rearing experiments began on 15 March 2015, several *P. lamellidens* larvae brought from their nest were introduced to each container as a potential food source for the microdontine larvae. Additionally, 80 adult workers were introduced to each container five days after rearing started. Furthermore, maple syrup diluted in water at a ratio of 1:1 and placed on a small piece of aluminum foil was supplied in each rearing container every three days.

Each rearing container was kept at approximately 25ºC in a darkroom. Observations of the animals were conducted at intervals of one to three days until they became adults. During this period, interaction behaviors between the microdontine larvae and the ants were recorded as well. Additionally, to better observe the ventral morphology of the larva, we detached one larva (No. 2) from a piece of bark flake and placed it on the side wall of the transparent container. The ventral surface of the larva is sticky enough to attach to the container wall. *P. lamellidens* workers were removed before the microdontine adults emerged to avoid any attack behavior of ant workers against adult hover flies (Akre et al., 1973; Wheeler, 1908).

2-2-3 Morphological studies

I conducted observations on the morphology of the microdontines at the larval and adult stages. The body color and morphology of the dorsal surface were recorded in individual larvae, prepupae [this stage lies between the moment when the cuticle of the larva hardens and the appearance of the anterior spiracles (Akre et al., 1973; Akre et al., 1988)], and pupae. Morphological observations of the puparium were conducted using all the individuals obtained, whereas only one individual emerged successfully and was used for morphological observation of the adult. The adult was prepared as a dry specimen after killing at -20ºC. The sample was kept in the freezer until subsequent morphological

observation for species identification was conducted. The puparium was detached from the surface of the piece of bark using invert soap. Morphological observations were conducted according to Maruyama & Hironaga (2004) for the adult and Thompson (1981), Rotheray (1993), and Oishi (1996) for the puparium. The anterior spiracles and posterior respiratory process of the puparium, and dorsal views of the adult and puparium were photographed with a Canon Eos D60 with an MP-E65 macro lens several times with differing focuses. These photographs were then synthesized by depth synthetic processing using the Combine ZP software (Hadley, 2010). The length, width, and height of puparium were measured using a vernier caliper. Furthermore, the height and width of both anterior spiracles and a marginal band were measured using the VHX-5000 system (Keyence, Osaka, Japan).

2-3 Results

2-3-1 Development of a microdontine species under rearing conditions

The microdontine larvae did not show any predatory behavior against *P. lamellidens* larvae throughout the rearing experiments. Also, no attack behavior of *P. lamellidens* workers against the microdontine larvae and pupae was observed. One of the larvae (No. 2) started pupation 24 days after collection in the field, and the other two (No. 1 and 3) began pupation 16 days after that (Table 2-1). Since no exuviae were found in the container prior to pupation of the larvae, the larvae were at the final instar stage at the time they were collected in the field. A pair of discs was observed on the dorsal front part of the individuals (Figure 2-6). The appearance of a pair of anterior spiracles from the discs, which is a signature of development from prepupa to pupa, was confirmed three days after the beginning of pupation in individuals No. 2 and eight days after the beginning of pupation in No. 1 and 3.

Individuals No. 1 and 3 emerged as adults 25 days after the beginning of pupation (Figure 2-7, Table 2-1). Both individuals failed to expand their wings, presumably because of a lack of an appropriate substrate for the individuals to mount on and expand their wings appropriately. These two died two days after their emergence.

I set a ladder-like scaffold made of an egg paper tray inside a corner of the container for individual No. 2 to mount on 17 days after pupation. In addition, the lid was removed from the container since I noticed that there was condensed water due to the high relative humidity on the inner surface of the container. Then, individual No. 2 emerged with fully

expanded wings. The adult of individual No. 2 was transferred into a small glass vial and killed by subjecting it to a temperature of -20ºC in a freezer six days after emergence.

Table 2-1. Days observed for each larva to reach pupal and adult stages after the collection of the specimens from their habitat.

Individuals	Pupation	Appearance of anterior spiracle	Emergence of adult
No. 1	16	24	41
No. 2	24	27	48
No. 3	16	24	41

Figure 2-6. A pair of discs of the prepupa. A pair of discs was observed on the dorsal front part.

Figure 2-7. A microdontine adult (individual No. 3) with eclosion insufficiency. It has unexpanded wings.

2-3-2 Morphology of larval, prepupal, pupal, puparial, and adult Microdontinae

Morphological observations of the microdontine species were conducted at different developmental stages. All the microdontine larvae had a dark brown reticulated structure with a pale green color on the overall dorsal surface (Figures 2-3 and 2-4). The ventral surface of the larva had an emerald green color (Figure 2-8). The whole-body color of the larva changed to light brown when it entered the prepupa stage (Figure 2-6). A uniform reticular structure was observed on the dorsal surface of the prepupae and pupae as well as the larvae (Figures 2-6 and 2-9). The body color of the pupa became dark brown three to eight days after the beginning of pupation (Figure 2-9).

Three pieces were made from the puparium shown on the left side in Figure 2-10 when the adult emerged. These puparia were 12.8–13.1 mm in length (n=3), 8.6–8.8 mm in width (n=3) and 6.0 mm in height (n=1: the heights of puparia No. 1 and No. 3 were not measured because the dorsal surfaces of these puparia were damaged). The thick marginal band consisted of both dorsal fringe and ventral fringe (Figure 2-11). The anterior spiracle on the right side was 398–450 μm in height and 464–488 μm in width (n=2: the right anterior spiracle of No. 1 was not measured because it was lost at the time of emergence) and that on the left side was 410–465 μm in height and 484–497 μm in width (n=3). In addition, protruding pores were observed on the surface of the anterior spiracles in each individual (Figure 2-12). The spiracular opening at the posterior respiratory process was separated into two areas by a flat median carina (Figures 2-13 and 2-14).

Concerning the adult, observation was conducted on the specimen cast by individual No. 2 only, which successfully emerged (Figure 2-15). The adult of individual No. 2 had a brilliant pale green color with metallic luster except for the caudal part of the abdomen, which was dark purple (Figure 2-15). This adult specimen was identified as a female of *M. katsurai* according to Maruyama & Hironaga (2004).

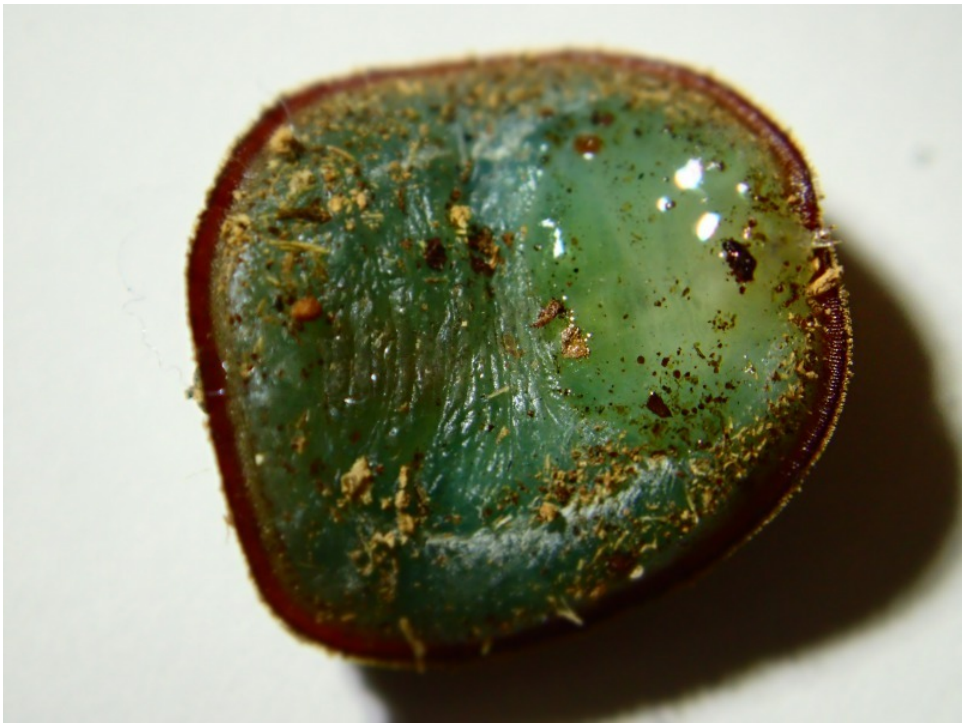


Figure 2-8. Ventral view of the *M. katsurai* larva (individual No. 2). The ventral surface of the larva had an emerald green color.

Figure 2-9. A pair of anterior spiracles protruded from the discs. A pair of anterior spiracles is refered to as “as”.

Figure 2-10. A microdontine puparium, dorsal view (individual No. 2). Pieces of the puparium (left) formed after eclosion.

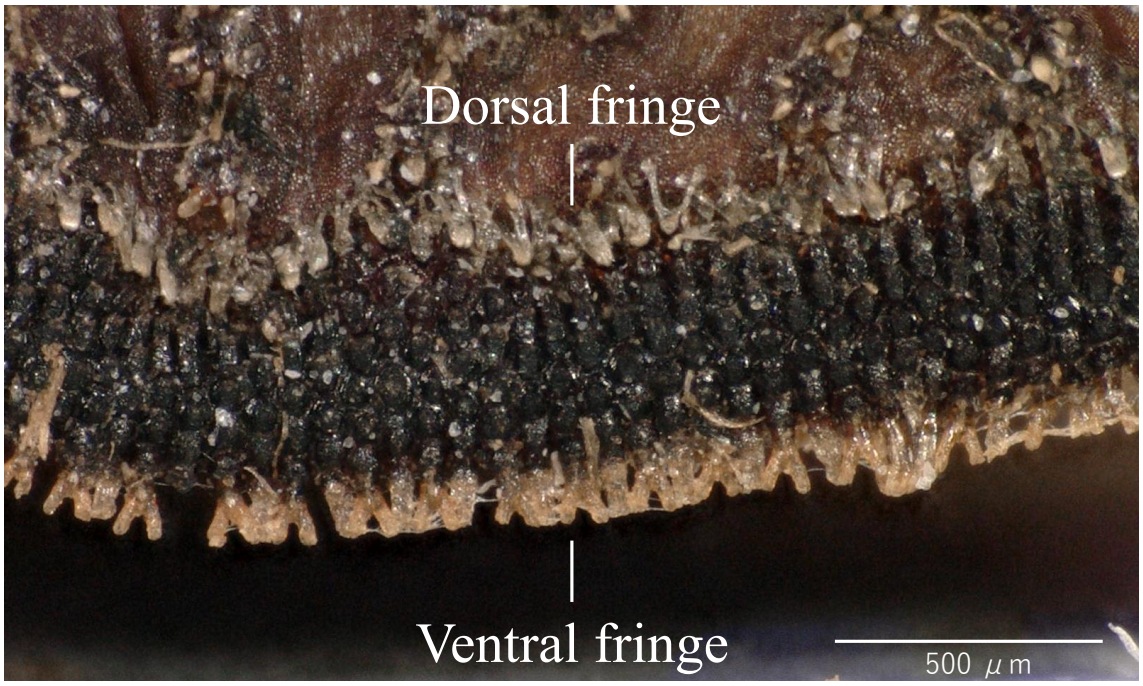


Figure 2-11. Lateral view of the puparium edge (individual No. 2). A dorsal fringe and a ventral fringe were observed.

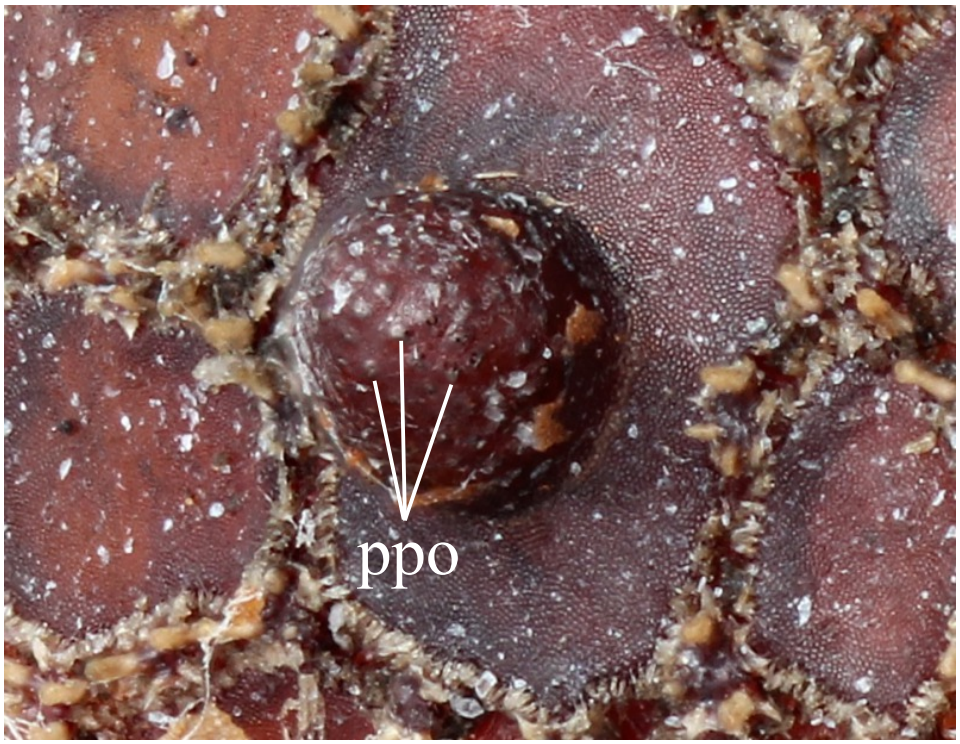


Figure 2-12. Anterior spiracle, lateral view (individual No. 2). Protruded pores is refered to as "ppo".

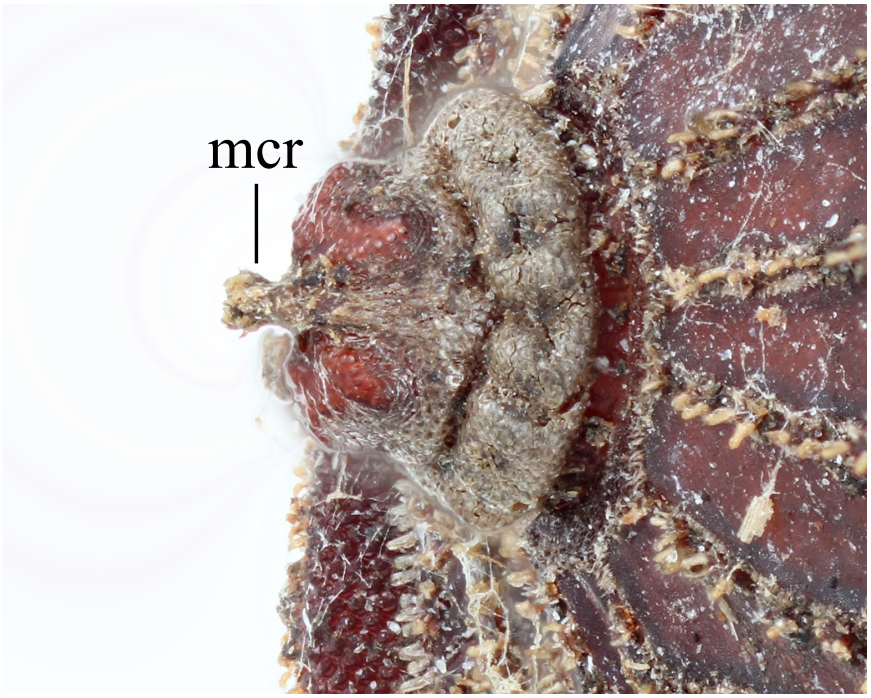


Figure 2-13. Posterior respiratory process, dorsal view (individual No. 2). Median carina is refered to as **"**mcr".

Figure 2-14. Posterior respiratory process, caudal view (individual No. 2). The spiracular opening was separated into two areas.

Figure 2-15. Dorsal view of the microdontine adult (individual No. 2). Individual No. 2 only emerged successfully.

2-4 Discussion

Based on observations of the appearance and ovipositional behavior of adult *M. katsurai* near *P. lamellidens* nests (Hironaga et al., 1998; Katsura, 1998; Maruyama & Hironaga, 2004), *M. katsurai* was speculated to spend its immature stages in *P. lamellidens* nests. In the present study, for the first time, I found *M. katsurai* larvae from the inside of a *P. lamellidens* nest and succeeded in rearing them to the adult stage when they were reared with *P. lamellidens* workers and larvae. This finding provides firm evidence that *M. katsurai* utilizes *P. lamellidens* nests. *M. katsurai* has never been found near nests of other ant species, suggesting that it is a host-specific myrmecophilous species.

P. lamellidens workers were not observed to inspect or attack the *M. katsurai* larvae in the rearing environment, suggesting that *M. katsurai* larvae can survive inside a *P. lamellidens* nest. Howard et al. (1990b) demonstrated that the cuticular chemical profiles of the larvae of the myrmecophilous Microdontinae species *Microdon albicomatus* Novak 1977 are the same as that of its prey, the pupae of the ant *Myrmica incompleta* Provancher 1881. *M. katsurai* larvae might also employ such chemical mimicry for the avoidance of attacks by *P. lamellidens*, although no data on cuticular chemical profiles in this species are available. I cannot conclude that the *M. katsurai* larvae grew by preying on *P. lamellidens* because they were not observed to prey on or attack *P. lamellidens* adults or larvae during the rearing experiments. Nevertheless, all the *M. katsurai* larvae pupated and emerged (Table 2-1). Thus, it is obvious that these larvae had fed on some food source sufficient for them to grow into adults before I had collected them. It has been reported that larval microdontines prey on ant eggs, larvae, and pupae (Duffield, 1981; Garnett et al., 1985; Pérez-Lachaud et al., 2014; Reemer, 2013; Van Pelt & Van Pelt, 1972). Others have suggested that the larvae of certain Microdontinae feed on refuse or pellets of food ejected by ants (e.g., Wheeler, 1908), but such feeding behavior has never been confirmed (Reemer, 2013). Further studies in both the field and laboratory are required to reveal the types of food resources *M. katsurai* larvae utilize.

Having been collected in March, the stage of all the larvae was likely the last instar because they did not molt before becoming pupae. Adult *M. katsurai* appears from the end of May until the beginning of July (Hironaga et al., 1998; Katsura, 1998; Maruyama & Hironaga, 2004), suggesting that this species overwinters in an ant nest before pupation. The three individuals studied emerged between 25 April and 2 May 2015 under laboratory

rearing conditions. The time of emergence in the present study was almost one month earlier than the recorded observations for adults of this species in the field (Maruyama & Hironaga, 2004). This difference in appearance time may be due to the rearing temperature (25ºC) in the present study, which is higher than that of the natural habitat of this species.

The color of larvae of microdontine species is white or brown (Dixon, 1960; Greene, 1955; Maruyama et al., 2013; Rotheray, 1991; Schmid et al., 2014; Wheeler, 1908 & 1924). The pale green color shown on the dorsal surface of the *M. katsurai* larvae in this study is rare in Microdontinae; the larvae of most species in this subfamily show a white or brown color (Dixon, 1960; Greene, 1955; Maruyama et al., 2013; Rotheray, 1991; Schmid et al., 2014; Wheeler, 1908 & 1924). This is the first record in Asia of a microdontine larva with such a remarkable color.

Based on morphological studies of adult specimens, *M. katsurai* is classified into the subgenus *Chymophila* (Reemer & Stahls 2013). My morphological studies on the *M. katsurai* puparium support this classification. There are several morphological similarities between the *M. katsurai* puparium and that of *Microdon* (*Chymophila*) *fulgens*, such as dorsal surfaces that have a uniform reticular structure, a thick marginal band consisting of both a dorsal fringe and a ventral fringe, and anterior spiracles with a height that is shorter than its width (Thompson, 1981). Moreover, in the puparium of both species, a flat median carina divides the two areas of the opening of the spiracles at the spiracular plate of the posterior respiratory process, which is characteristic of the subgenus *Chymophila* (Thompson, 1981). Considering all this together, the classification by puparium morphology in the present study is concordant with that of adult morphology.

The two individuals that failed to emerge, No. 1 and No. 3, could not be identified using adults, but they are considered to be *M. katsurai* based on the morphology of their puparia.

In Japan, thus far, *M. katsurai* has been recorded in the Nagano, Ibaraki, Tochigi, Mie, Osaka, Hyogo, Yamaguchi, Kagawa, and Kagoshima prefectures (Genka, 2010; Hironaga et al., 1998; Iwai, 2010; Kano, 1999; Katsura, 1998; Kusama & Tamaki, 2004; Noishiki, 2014; Sadahiro, 2013; Tanaka, 2002), and for the first time, it was collected in the Yamanashi prefecture in the present study. Although *M. katsurai* is often recorded in lowland environments (Maruyama & Hironaga, 2004), it has also been found in habitats with relatively high altitudes of up to 600 m (Noishiki, 2014). The altitude of the habitat

where *M. katsurai* was found in the present study is approximately 600 m, suggesting that the habitat preference of this species is broader than previously speculated.

M. katsurai is a rare species that is designated as Vulnerable (VU) in Japan (Ministry of the Environment, 2015). Furthermore, the habitat of *P. lamellidens*, which is designated as VU as well, is decreasing from the influence of recent residential and industrial development (Maruyama & Hironaga, 2004; Maruyama et al., 2013; Ministry of the Environment, 2015; Sadahiro, 2013). The present study indicated a strong ecological association between *M. katsurai* and *P. lamellidens*. It is necessary to protect the *P. lamellidens* habitats immediately to conserve not only *P. lamellidens* but also the very rare species *M. katsurai*.

Chapter 3

The evidence of temporary social parasitism by *Polyrhachis lamellidens* (Hymenoptera, Formicidae) in a *Camponotus obscuripes* colony (Hymenoptera, Formicidae)

3-1 Introduction

P. lamellidens is a temporary social parasite that establishes new colonies with the assistance of a host species (Furukawa et al., 2012; Japanese Ant Database Group, 2003; Kohriba, 1963 & 1966; Kubota, 1974; Sakai, 1996 & 2000; Yano, 1911) and is a known host of myrmecophiles (Iwai et al., 2016). *P. lamellidens* queens allow their host workers to care for their parasitic brood (eggs, larva, and pupa), resulting in a temporary period during which host-reared *P. lamellidens* workers and host workers inhabit the same colony (Japanese Ant Database Group, 2003; Kohriba, 1963; Sakai, 2000). This mixed colony transitions to a *P. lamellidens*-only colony as the number of host workers gradually decreases (Japanese Ant Database Group, 2003). Several species (*C. japonicus*, *C. obscuripes*, and *C. kiusiuensis*) have been suggested to be hosts of *P. lamellidens* (Furukawa et al., 2012; Japanese Ant Database Group, 2003; Kohriba, 1963 & 1966; Kubota, 1974; Sakai, 1990, 1996, & 2000; Yano, 1911). *C. japonicus* has been confirmed as a host species according to observations of mixed colonies of *P. lamellidens* and *C. japonicus*. In mixed colonies in the field, the two different species coexisted without any aggressive behavior towards one other (Kohriba, 1966). In addition, Kohriba demonstrated the early conditions of parasitic life in a laboratory environment and even observed brood production by *P. lamellidens* (Kohriba, 1963).

There have been reports of new queens of *P. lamellidens* exhibiting an initial performance (the behavior of straddling host workers, which is speculated to promote invading the host colony) towards *C. obscuripes* workers and inhabiting *C. obscuripes* colonies (Furukawa et al., 2012; Sakai, 1996). These findings suggest that *C. obscuripes* serves as a host for the temporary social parasite *P. lamellidens*. However, to accept this hypothesis, the following three factors must be examined and confirmed: parasite brood production, nestmate discrimination, and parasitism style. Parasite brood production is a primary event required to complete the social parasitism cycle (Buschinger, 2009; de la Mora et al., 2020; Hölldobler & Wilson, 1990). If *C. obscuripes* serves as the host, then *P. lamellidens* workers and brood must be identified along with *C. obscuripes*. However, *P. lamellidens* workers and brood have not yet been found in *C. obscuripes* colonies in the field or under artificial rearing conditions. Nestmate discrimination, i.e., whether *C. obscuripes* workers accept the parasites as nestmates must also be verified. Since a parasite and its host inhabit the same colony, they must recognize each other as nestmates while exhibiting aggressive behavior towards non-nestmates. Furthermore, it is necessary

to show that *P. lamellidens* utilizes *C. obscuripes* as a host for temporary social parasitism and not other types of parasitism. In temporary socially parasitic ants, such as *P. lamellidens* and *C. japonicus* mentioned above, temporary coexistence between parasites and hosts occurs after killing the host queen.

The coexistence of parasites and hosts has also been shown in other social parasite strategies, such as xenobiosis and inquilinism, but these strategies do not result in the killing of a host queen by the parasite (Buschinger, 2009; de la Mora et al., 2020; Hölldobler & Wilson, 1990). For example, *P. lama* (Formicidae: Formicinae) and *P. loweryi* (Formicidae: Formicinae) have been identified as socially parasitic species (xenobiosis) of *Polyrhachis* species other than *P. lamellidens* (Maschwitz et al., 2000, 2003), and they do not kill the host queen. Dulotic species also kill the host queen; however, the subsequent coexistence with the host colony is permanent (because host workers are continually supplied by slave raiding), and they do not eventually replace the colony as some temporary socially parasitic ants do (Buschinger, 2009; de la Mora et al., 2020; Hölldobler & Wilson, 1990). Dulotic species are strictly divided into obligatory dulosis and facultative dulosis, in which the former worker lacks the working ability while the latter possesses it. If *P. lamellidens* utilizes *C. obscuripes* as a host, then it is necessary to show that the *C. obscuripes* queen is killed by the *P. lamellidens* queen and that *P. lamellidens* workers can survive in the absence of their host. Therefore, exploration of mixed colonies in the field, behavioral tests, and rearing experiments are necessary to show that *C. obscuripes* is a host of temporary social parasites.

In this chapter, I report the first record of a mixed colony consisting of *C. obscuripes* workers and a *P. lamellidens* queen, workers, and brood from Yamagata Prefecture, Japan. Two independent mixed colonies were found in the field, and the symbiotic relationship was confirmed by behavioral tests. I also confirmed through rearing experiments that *P. lamellidens* queens practice social parasitism against *C. obscuripes*. These observations indicate that *P. lamellidens* can complete the social parasitism cycle using *C. obscuripes* as a natural host.

3-2 Materials and Methods

3-2-1 Sampling and rearing

Ant colony samples were collected in Yamagata and Niigata Prefecture, Japan (2019 to 2020). Two mixed colonies of *P. lamellidens* and *C. obscuripes* were sampled from a

primeval beech forest in Oguni-machi, Yamagata Prefecture (N 37°55′13″, E 139°40′54″; N 37°55′24″, E 139°40′27″) and named colony A and B, respectively. Each colony (A:B) consisted of a *P. lamellidens* queen, a worker, a larva, and a *C. obscuripes* worker in the following numbers: 1:1, 729:12, 200:244, and 31:112. None included the queen and larva of *C. obscuripes* (Table 3-1). The *P. lamellidens* and *C. obscuripes* workers used in the behavioral test and the rearing experiment were collected from free-living colonies. Two free-living *C. obscuripes* colonies not parasitized by *P. lamellidens* were collected from a primeval beech forest in Oguni-machi (N 38°08′48″, E 139°50′35″) and a mixed forest in Tsuruoka City, Yamagata Prefecture (N 38°33′49″, E 139°55′31″); the average colony size consisted of one queen and 300 workers. One free-living *P. lamellidens* colony was collected from mixed forests (N 38°41′43″, E 139°48′2″); the colony size consisted of 20 workers. Eight newly mated *P. lamellidens* queens were collected after finishing their nuptial flights from a mixed forest in Nagaoka City, Niigata Prefecture (N 37°25′51″, E 138°52′53″). Each collected ant colony was reared separately in a plastic tub (10.0 cm in length, 10.0 cm in width, 2.9 cm in height, or 27.0 cm in length, 19.0 cm in width, 5.1 cm in height) in which plaster was laid and a feeding area was established or in a plastic cup (9.5 cm in diameter, 4.5 cm in height) in which moistened tissue was laid. Newly mated *P. lamellidens* queens were reared individually in a plastic tub that was 4.5 cm in length, 2.5 cm in width, and 2.0 cm in height and contained moistened tissue. The ants were fed 5–5000 μl of 50% maple syrup (Maple Farms Japan, Limited, Osaka, Japan) every four to ten days. Worker ants were also fed mealworms and/or 8–16 g of Pro Jelly for beetles and stag beetles (Wraios, Limited, Saitama, Japan). Mixed colonies and non-mixed colonies (*C. obscuripes* and *P. lamellidens*) were reared under either dark conditions at 15–25°C or light–dark conditions (14L:10D) at 20–30°C. The colonies experimentally invaded by *P. lamellidens* were reared in a cool incubator (Mitsubishi Electric Engineering Company, Limited, Tokyo, Japan) under dark conditions at 20–25°C. Newly mated *P. lamellidens* queens were reared in a cool incubator under dark conditions at 15–20°C. The identifications were performed based on morphological characteristics (referring to Japanese Ant Database Group, 2003) and the mitochondrial genes encoding cytochrome oxidase subunits I and II (COI/II) in the NCBI database (http://www.ncbi.nlm.nih.gov). DNA was extracted from the legs by crushing them with a disposable homogenizer and treating them with proteinase K. Extracted DNA was amplified by polymerase chain reaction (PCR) with the following conditions: 98°C for 2

min, 30 cycles each of 98°C for 10 s, 55°C for 15 s, and 72°C for 2 min with LifeECO Thermal Cycler (Bioer, Zhejiang, China). The Sanger sequencing of the amplified DNA was performed by Eurofins Genomics (Tokyo, Japan). C1-J-1754F (CCACGTTTAAATAATATAAGATTTTGAC) and C2-N-3661R (CCACAAATTTCTGAACATTGACCA) were used as PCR primers (Degnan et al. 2004; Simon et al. 1994). Sequences from *P. lamellidens* and *C. obscuripes* from mixed colonies and free-living were confirmed by matching each species by alignment and clustering with MAFFT v7 (Kuraku et al., 2013).

3-2-2 Behavioral tests

Behavioral tests were conducted in a plastic tub (8.0 × 4.0 × 3.0 cm), with one worker ant from each colony (the mixed Colony A, the free-living *C. obscuripes* colony, and the free-living *P. lamellidens* colony) encountering others. Mixed colony B was not used for behavioral tests because of the low number of *P. lamellidens* workers. The ants were selected at random and used only once in each test. The workers used for the test were transferred to another case and were not duplicated in subsequent tests. For 30 s, after introducing each ant into the case, a partition was used to prevent contact, reduce stress, and calm their behavior. The first behavior noted during the first contact in less than 6 min after the removal of the partition was recorded according to the following definitions:

Ignore: the ant does not respond aggressively to contact with the target.

Threat: the ant opens its mandibles or keeps its distance from the target.

Rush: the ant approaches and quickly bites the target.

Bite: the ant continuously bites the target.

Among these behaviors, “Threat”, “Rush”, and “Bite” were considered aggressive behaviors.

3-2-3 Rearing experiments involving temporary social parasite

The study of temporary social parasitism in a laboratory environment was conducted based on previously reported procedures (Kohriba, 1963). Assays to study initial contact between a newly mated *P. lamellidens* queen and a *C. obscuripes* worker were performed in well-ventilated tubes (1.0 × 8.0 cm). For eight newly mated *P. lamellidens* queens, one to twenty free-living *C. obscuripes* workers were placed in the tube every other day. After several days, when contact with more than 60 *C. obscuripes* workers was observed, the

P. lamellidens queen was transferred to an empty plastic tub without the *C. obscuripes* queen (10.0 × 10.0 × 2.9 cm). Once it was confirmed that the *C. obscuripes* workers were not attacking the transferred *P. lamellidens* queen, she was allowed to contact the *C. obscuripes* queen. The *C. obscuripes* queen was subjected to anesthesia by freezing (4°C for 2 min, -20°C for 10 min) before contact with a new *P. lamellidens* queen. A newly mated *P. lamellidens* queen is often attacked by the *C. obscuripes* workers or queens, as observed in rearing experiments in previous studies (Sakai, 1996 & 2000). Since the purpose of this rearing experiment was to learn whether the newly mated *P. lamellidens* queen would recognize the *C. obscuripes* queen as her host, to exclude the possibility of counterattack by the *C. obscuripes* queen, I conducted the anesthesia treatment.

3-3 Results

3-3-1 Field observations and mixed colony composition

Two mixed *P. lamellidens* and *C. obscuripes* colonies were found inside fallen beech trees in Yamagata, Japan. A queen, larvae, and workers of *P. lamellidens* and workers of *C. obscuripes* coexisted in the same colony (Figure 3-1A, Table 3-1). The balance of worker numbers between *P. lamellidens* and *C. obscuripes* was biased towards *P. lamellidens* in Colony A and *C. obscuripes* in Colony B (Table 3-1). I used only Colony A in the behavioral test because the number of *P. lamellidens* workers at Colony B was small. Only one *P. lamellidens* queen was found in each colony. In addition, the *P. lamellidens* queen found in Colony A possessed left and right mid and hind legs that were all severed at their bases (Figure 3-1B). After collection, no signs of mutual aggression were observed between the two species in the mixed colonies in the field and under laboratory conditions. Under laboratory-rearing conditions, trophallaxis between *C. obscuripes* and *P. lamellidens* workers was observed (Figure 3-1C). All *C. obscuripes* workers (host workers) belonging to Colony A died within approximately two months after collection. The *P. lamellidens* colony has persisted to the present day (as of October 2020). The ants in the colonies were identified as *P. lamellidens* and *C. obscuripes* by comparing the COI/II sequences of non-parasitized *C. obscuripes* and free-living *P. lamellidens*.

Table 3-1. Compositions of the two mixed colonies.

	P. lamellidens			*C. obscuripes*		
	Queen	Workers	Larvae	Queen	Workers	Larvae
Colony A	1	729	>200	0	31	0
Colony B	1	12	244	0	112	0

Figure 3-1. The mixed colony of *P. lamellidens* and *C. obscuripes*. (A) *P. lamellidens* and *C. obscuripes* were collected from the same colony (A: Colony A). (B) *P. lamellidens* queen isolated from Colony A. This queen's left and right mid and hind legs were all severed at the base. (C) *P. lamellidens* and *C. obscuripes* perform trophallaxis (Colony A).

3-3-2 Behavioral tests

Behavioral tests were performed to quantitatively determine whether the mixed colony composition was a result of temporary social parasitism by *P. lamellidens*. When *C. obscuripes* and *P. lamellidens* workers from the same mixed colony contacted one another, no aggressive behaviors were observed (Table 3-2). To exclude the possibility that these workers obtained from the mixed colonies were deficient in the ability to recognize their nestmates, each worker was placed in a container with a worker from a different unparasitized colony. Both species showed clear aggressive behaviors towards the other species (Table 3-2). These results indicate that *P. lamellidens* and *C. obscuripes* inhabiting a mixed colony are able to recognize each other as nestmates.

3-3-3 Artificial social parasitism rearing experiment

To observe the initial stages of parasitism by *P. lamellidens* among *C. obscuripes* in a laboratory environment, I conducted rearing experiments. All newly mated *P. lamellidens* queens collected from the field were reared in the laboratory for more than six months. First, I allowed eight newly mated *P. lamellidens* queens to contact non-parasitized *C. obscuripes* workers in a well-ventilated tube individually. As in previous studies, the newly mated *P. lamellidens* queen straddled the host worker and rubbed their body with their legs (Japanese Ant Database Group, 2003; Kohriba, 1963; Kubota, 1974; Sakai, 1990 & 2000). Although no quantitative records exist, some newly mated *P. lamellidens* queens died while contacting the host workers. As a result of this phenomenon that appears to host's attack, five out of eight newly mated *P. lamellidens* queens survived the contact process with *C. obscuripes* workers. After sufficient contact with *C. obscuripes* workers, one of the five surviving newly mated the *P. lamellidens* queens met the non-parasitized *C. obscuripes* queen. The newly mated *P. lamellidens* queen immediately bit the *C. obscuripes* queen's neck (Figure 3-2A). This biting behavior continued for three days, and eventually, the *C. obscuripes* queen's head was severed (Figure 3-2B). This killing behavior against the *C. obscuripes* queen was distinctly different from common bite attacks (as described in "Materials and Methods of Chapter 3"). The newly mated *P. lamellidens* queen started laying eggs four days after killing the *C. obscuripes* queen (Figure 3-2C). More than ten eggs were confirmed within a week of the first oviposition. *P. lamellidens* eggs were collected and cared for by *C. obscuripes* workers (Figure 3-2D). Between the killing of the host queen and oviposition, *C.*

obscuripes workers did not show any aggressive behaviors towards the newly mated *P. lamellidens* queen.

Table 3-2. The presence of aggressive behaviors in each colony (n=10).

		P. lam **(Mixed) vs** ***C. obs*** **(Mixed)**	***P. lam*** **(Normal) vs** ***C. obs*** **(Normal)**	***P. lam*** **(Mixed) vs** ***C. obs*** **(Normal)**	***P. lam*** **(Normal) vs** ***C. obs*** **(Mixed)**
Aggressive behavior	**Threat**	0	9	4	1
	Rush	0	1	5	6
	Bite	0	0	0	3
Non-aggressive behavior	**Ignore**	10	0	1*	0

All tests showed common tendencies in both species to initiate or not initiate aggressive behavior (*: In only one out of ten tests, aggressive behavior was initiated on the second contact, not the first). I used a mixed colony (Colony A) and normal colonies without hosts or parasites (*P. lamellidens*, *C. obscuripes*) in the behavioral tests. *P. lam*: *P. lamellidens*, *C. obs*: *C. obscuripes*.

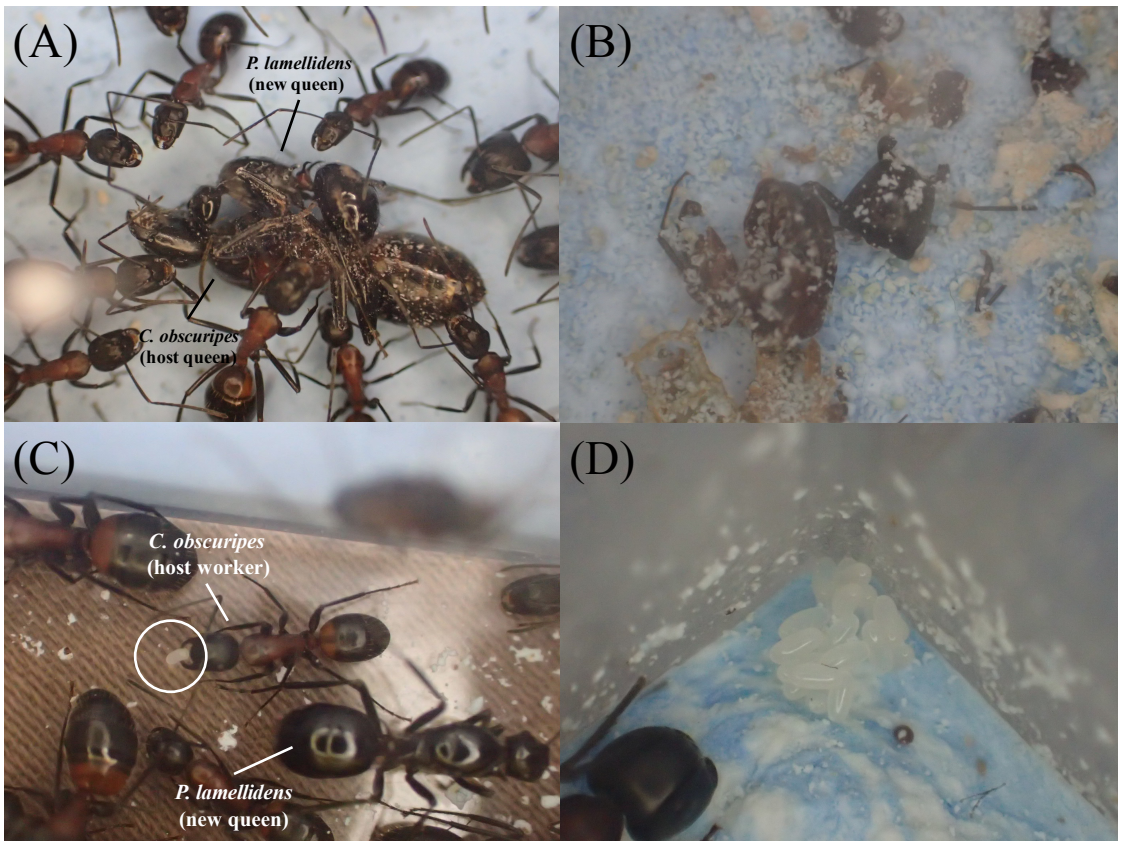


Figure 3-2. The early stage of the parasitism cycle. (A) The new *P. lamellidens* queen bites the neck of the *C. obscuripes* queen. (B) The *C. obscuripes* queen after being attacked for three days. (C) Eggs laid by the parasite queen (*P. lamellidens*) in the host (*C. obscuripes*) colony. *P. lamellidens* eggs were laid after the killing of the *C. obscuripes* queen. (D) *P. lamellidens* eggs were gathered by *C. obscuripes* workers.

3-4 Discussion

C. obscuripes has been suggested as a possible host for *P. lamellidens* (Furukawa et al., 2012; Sakai, 1996), but there was no conclusive evidence. This study is the first record of a mixed colony of *C. obscuripes* and *P. lamellidens* in the field, and *P. lamellidens* parasitism of *C. obscuripes* colonies was confirmed in a rearing experiment.

In the behavioral tests, *P. lamellidens* and *C. obscuripes* workers from the same mixed colony showed aggressive behavior towards workers from other colonies. Nevertheless, they recognized each other as nestmates, even though they were different species (Table 3-2). However, non-parasitized *C. obscuripes* exhibited distinct aggressive behaviors towards *P. lamellidens*. These results suggest that both species inhabit the mixed colony as nestmates. It is thought that a newly mated *P. lamellidens* queen will kill the host queen in the early stages of social parasitism according to the previous knowledge of *C. japonicus* as its host (Japanese Ant Database Group, 2003). I carried out rearing experiments to verify that *C. obscuripes* is affected by social parasitism. In my rearing experiment, I observed that the *P. lamellidens* queens started laying eggs after killing the host queen (Figure 3-2). Thus, I anesthetized the *C. obscuripes* queen before contacting the newly mated *P. lamellidens* queen in the rearing experiment. However, since the newly mated *P. lamellidens* queen demonstrated the same killing process toward *C. japonicus*, as shown only in the case of a *C. obscuripes* queen in previous studies (Japanese Ant Database Group, 2003), I believe anesthesia does not affect the hypothesis verification of this experiment. Host-queen killing and oviposition in the host colony are common features of temporary socially parasitic and dulotic ants, not xenobiosis and inquilinism (Buschinger, 2009; de la Mora et al., 2020; Hölldobler & Wilson, 1990). *P. lamellidens* persisted after the death of the host workers in mixed Colony A. Temporary coexistence with the host and sustaining the colony after the host's death are unique characteristics in temporary socially parasitic ants, not dulotic ants (with some exceptions, e.g., facultatively dulotic ants) (Buschinger, 2009; de la Mora et al., 2020; Hölldobler & Wilson, 1990). Based on these observations, the two colonies obtained from the field provide evidence of *P. lamellidens* completing the temporary social parasitism cycle in a *C. obscuripes* colony, and I have clear evidence that *C. obscuripes* is a natural host for *P. lamellidens*. The above experiments and observations verified that *P. lamellidens* is not an obligatorily dulotic ant, as they could persist after the death of the host workers. However, the possibility of facultative dulosis has not yet been rejected. None of the

reports of raiding behavior in the field or a few records of the mixed colony with its hosts suggests that *P. lamellidens* is an unlikely facultatively dulotic ant; thus, further verification is needed.

The balance of worker numbers in the two mixed colonies was different (Table 3-1). I speculate that this reflects the difference in the time that has elapsed since the host queen was killed. Probably, Colony A had been around longer than Colony B since the killing of the host queen; therefore, it had fewer workers of *C. obscuripes* than *P. lamellidens*. The left and right mid and hind legs of the *P. lamellidens* queen in Colony A were all severed at their bases (Figure 3-1B). In past fieldwork (unpublished) in another region [Nirasaki City, Yamanashi Prefecture (N 35°42′49″, E 138°29′12″)], I observed an injured *P. lamellidens* queen from a free-living *P. lamellidens* colony. After the nuptial flight, a newly mated *P. lamellidens* queen invades a host colony to establish a new colony, but she is often attacked by some host workers at first contact, as observed in rearing experiments in previous studies (Kohriba, 1963; Sakai, 1996 & 2000). In this rearing experiment, a newly mated *P. lamellidens* queen, probably killed by an attack from its host workers, has been confirmed. I believe that the *P. lamellidens* queen of Colony A succeeded in usurping the host colony before being killed by the attack by host workers in the early stages of social parasitism.

C. obscuripes workers, members of the collected mixed colony, and those whose queen was killed in the rearing experiment did not show aggressive behaviors towards *P. lamellidens*, and both species coexisted as nestmates. This phenomenon suggests that *P. lamellidens* may have some involvement in nestmate discrimination by *C. obscuripes*. It is known that some socially parasitic ants alter host-like CHC profiles or express almost no CHCs, which promotes coexistence with their host (Akino, 2008; de la Mora et al., 2020; Dettner & Liepert, 1994; Johnson et al., 2001; Lenoir et al., 2001). *P. lamellidens* queens and workers in mixed colonies may utilize similar strategies. The behavior of straddling host workers was observed in *P. lamellidens* queens. As a previous study speculated (Kohriba, 1963), *P. lamellidens* queens may disguise their CHC profiles by performing this behavior, enabling invasion of the host colony.

The host *Diacamma* sp. and *Rhytidoponera* sp. workers (Formicidae, Ponerinae) of *P. lama* and *P. loweryi* do not rear parasites' brood (Maschwitz et al., 2003; Witte et al., 2009). It is speculated that the reason for the lack of brood care by the host is that the parasite/host is distantly related at the subfamily level, and there are discrepancies in

larval feeding methods (Formicinae larvae receive liquid nourishment that is preprocessed by workers, whereas Ponerinae larvae are provided unmodified prey particles by workers); thus, the rearing style of the host species is not compatible with the parasite brood (Witte et al., 2009). Conversely, in the case of *P. lamellidens*, the hosts *C. japonicus* and *C. obscuripes* were observed rearing *P. lamellidens* brood (Kohriba 1963) (Figures 3-2CD). *P. lamellidens* and its host ants are relatively closely related, and although they differ at the genus level, they both belong to the subfamily Formicinae (Brady et al., 2006) and probably share a common feeding method. The differences in the presence or absence of brood care by the host, even among socially parasitic species in the genus *Polyrhachis*, may be related to common brood feeding methods between parasites and hosts, as described above.

The nesting styles of *P. lamellidens* and its host differ. Initial nesting sites of *P. lamellidens* (*P. lamellidens* changes its nesting site at the end of the social parasitism cycle) are tree hollows, while *C. japonicus* nests in soil and *C. obscuripes* nests in decaying wood (Japanese Ant Database Group, 2003). Thus, *P. lamellidens* is relatively unusual as a socially parasitic ant. The exact explanation for why *P. lamellidens* utilizes *Camponotus* as its host is unclear, but *P. lamellidens* likely has high adaptability to various environments that harbor *Camponotus*.

Morphological similarities (e.g., body size) between socially parasitic ants and their hosts have been reported; one explanation for this phenomenon is that a socially parasitic ant has to adapt to the host's colony environment (e.g., tunnel size) (Fischer et al., 2020). This phenomenon is probably also true for *P. lamellidens* and its hosts. The workers of ants in the subgenus *Camponotus* (*C. japonicus* and *C. obscuripes* belong to this subgenus) are 7–12 mm long, which is similar to or longer than *P. lamellidens* (7–9 mm for workers, 10 mm for the queen) (Japanese Ant Database Group, 2003; Kohout, 2014). Conversely, workers of the subgenera *Myrmamblys* and *Myrmentoma* (belonging to the genus *Camponotus*), which are not hosts of *P. lamellidens*, are less than 5 mm in total body length (Japanese Ant Database Group, 2003).

This study revealed that *P. lamellidens* utilizes multiple ant species as a host (*C. japonicus* and *C. obscuripes*). In addition to *P. lamellidens*, socially parasitic ants that parasitize numerous hosts (e.g., *Lasius umbratus*) are known regardless of parasitism style. The common feature of these species is that the socially parasitic ants and their hosts tend to be closely related species, and the hosts belong to a specific taxonomic group

at the subfamily level (de la Mora et al., 2020; Emery, 1909; Hölldobler & Wilson, 1990; Huang & Dornhaus, 2008; Japanese Ant Database Group, 2003). The reason why the hosts are closely related to the parasitic ants and are restricted to few taxa is thought to be because it is easy for the parasitic ants to disguise their CHC profile (Blatrix & Sermage, 2005; de la Mora et al., 2020; Huang & Dornhaus, 2008); the same is probably true for *P. lamellidens*. Moreover, as mentioned, another factor in establishing social parasitism is that the host is closely related to the parasite species and can take care of the parasite's brood.

My findings provide evidence, for the first time, of temporary social parasitism involving *P. lamellidens* and *C. obscuripes*. The methodology presented in this investigation will contribute to, and accelerate, the study of social insects.

Chapter 4

Molecular evidence of chemical disguise by the socially parasitic spiny ant *Polyrhachis lamellidens* (Hymenoptera: Formicidae) when invading a host colony

4-1 Introduction

There are several hydrocarbons present on ant cuticles, and these hydrocarbons function as semiochemicals (Detrain et al., 1999; Howard & Blomquist, 2005; Sturgis & Gordon, 2012). The CHC profile of ants varies among different colonies. Ants discriminate between nestmates and foreign enemies (non-nestmates) by recognizing the differences in CHC profiles and behave aggressively towards individuals with different CHC profiles, even if the non-nestmates are the same species. On the other hand, solitary organisms that evade the nestmate discrimination by ants and utilize the workforce of ants are referred to as "myrmecophiles" (Kistner, 1982). In addition, social insects with strategies similar to myrmecophiles are referred "social parasites" (Buschinger, 2009). It is known that some myrmecophile species disguise their own CHC profile to obtain a similar profile to that of the host. This strategy is called "chemical disguise" (e.g., Hojo et al., 2009; Nehring et al., 2016). It is believed that myrmecophiles and socially parasitic ants utilize chemical disguises to avoid being exclusively discriminated as non-nestmates by host ants (Akino, 2008; Dettner & Liepert, 1994; Lenoir et al., 2001). For example, when a newly mated queen in the slave-making parasitic ant *Polyergus* (Hymenoptera: Formicidae) enters a host colony, she temporarily avoids aggressive behaviors from the host worker by emitting a repellent substance, then she kills the host queen, disguises her CHC profile, and exhibits the CHC profile of the host queen (d'Ettorre & Errard, 1998; d'Ettorre et al., 2000; Johnson et al., 2001; Mori et al., 1995; Tsuneoka, 2008; Tsuneoka & Akino, 2009 & 2012; Zimmerli & Topoff, 1994).

There are two methods of chemical disguise, including the chemical camouflage and chemical mimicry methods (Akino, 2008; Dettner & Liepert, 1994; Lenoir et al., 2001). Howards defined that organisms utilize the methods of "chemical camouflage" to acquire the CHC profile from the host directly, and "chemical mimicry" is utilized to biosynthesize host-like CHC (Howard et al., 1990a & b). Some studies have experimentally verified the mechanism of chemical disguise in several myrmecophile species, such as spiders and crickets (Akino et al., 1996; Howard et al., 1990b; Scarparo et al., 2019; von Beeren et al., 2011 & 2012; Vander Meer & Wojcik, 1982). The mechanism of chemical disguise was estimated in socially parasitic ants through ecological observations (Bauer et al., 2010; Lenoir et al., 1997). However, there is little information on the quantitative and molecular mechanisms that describe the intricacies of chemical disguise. Since the strategy of chemical disguise is a feature that is unique to

socially parasitic ants, I believe that elucidating the molecular basis of the detailed mechanisms will elucidate the evolutionary history of social parasitism.

P. lamellidens is an appropriate species to utilize when investigating the detailed mechanism of chemical disguise. There are approximately 700 species in the genus *Polyrhachis* (Bolton, 2022), of which three socially parasitic species are included. *P. lama* and *P. loweryi* are xenobiosis (Maschwitz et al. 2000, 2003, & 2009; Witte et al. 2009), whereas *P. lamellidens* is the only temporary socially parasitic ant species in the genus *Polyrhachis*. *P. lamellidens* utilizes several species of hosts, including *C. japonicus* and *C. obscuripes*, and the presumed candidate host, *C. kiusiuensis* (Iwai et al., 2021; Japanese Ant Database Group, 2003; Kohriba, 1963 & 1966; Kubota, 1974; Kurihara et al., 2022; Sakai, 1990, 1996, & 2000; Yano, 1911). These host *Camponotus* species overlap habitat with the initial nesting sites of *P. lamellidens* [(this species changes its nesting site at the end of the social parasitism (Japanese Ant Database Group, 2003; Kohriba, 1966)], and are common species here (Japanese Ant Database Group, 2003). As morphological similarity (e.g., body size) of parasite between its host has been pointed out as the recruitment of social parasitism (Fischer et al., 2020), *C. japonicus* and *C. obscuripes* are the only species of similar body size in the habitat of *P. lamellidens* (Japanese Ant Database Group, 2003). The social parasitism of *P. lamellidens* begins with the invasion of the host nest by the newly mated queen and the murder of the host queen. After a successful killing of the host queen, the newly mated queen starts oviposition, and all eggs are raised by host workers. As a result, all the new larvae are only *P. lamellidens*, and the host workers will continue to work for *P. lamellidens* until they die. Eventually, the colony will consist of only *P. lamellidens* (Iwai et al., 2021; Japanese Ant Database Group, 2003; Sakai, 1996 & 2000). In this species, the newly mated queen performs distinct behaviors when invading the host colony, and these behaviors include straddling the host worker and rubbing its body with her legs (Japanese Ant Database Group, 2003; Kohriba, 1963; Kubota, 1974; Kurihara et al., 2022; Sakai, 1990 & 2000) (Figure 4-1A). This behavior, which is known as a “rubbing behavior”, is unique to *P. lamellidens* queens that are newly mated, is considered a strategy of chemical disguise, and prevents attacks by the host (Kohriba, 1963); however, little is known about the molecular machinery of this strategy.

In this study, I quantitatively evaluated the efficiency of rubbing behavior-induced chemical disguise by measuring CHC profiles and conducting behavioral tests, and this

was performed to clarify the effect of rubbing behavior on the CHC of *P. lamellidens* and nestmate recognition of host workers. Furthermore, I elucidated the mechanisms of the disguise strategy of *P. lamellidens* by performing tracing assays with the labeled substances and by analyzing the expression of related genes, aiming to reveal the behavioral ecology, biochemistry, and molecular biology foundations that explain social parasitism in ants. Therefore, in this study, gas chromatography-mass spectrometry (GC-MS) analyses of CHC were performed to quantitatively evaluate the extent to which the rubbing behavior achieves chemical disguise. Through behavioral experiments, I examined the reactions of host workers to the chemical disguise obtained by rubbing behavior. Furthermore, by labeling assays and gene expression profiling, I investigated whether chemical disguise is implemented by camouflage or mimicry.

4-2 Materials and Methods

4-2-1 Sampling and rearing

Ant samples were collected in Niigata, Yamanashi, and Yamagata Prefecture, Japan (2015 to 2021). Approximately 50 newly mated *P. lamellidens* queens were collected from a mixed forest in Nagaoka City, Niigata Prefecture (N 37°25′51″, E 138°52′53″), and after the queens finished their nuptial flights, their original colony was not known. A *P. lamellidens* colony was collected from a primeval beech forest in Nirasaki City, Yamanashi Prefecture (N 35°42′49″, E 138°29′12″); the colony approximately consisted of three queens, more than 1,000 workers and larvae. *C. japonicus* and *C. obscuripes* colonies were collected from a primeval beech forest in Nagaoka City, Niigata Prefecture (N 37°41′42″, E 138°88′95″), a mixed forest in Tsuruoka City, Yamagata Prefecture (N 38°33′49″, E 139°55′31″), and a mixed forest in Minamikoma District, Yamanashi Prefecture (N 35°46′11″, E 138°29′43″); the average colonies consisted of 100 workers for *C. japonicus* and one queen and 200 workers for *C. obscuripes*. The host workers used in each experiment were from the same colony each time.

The rearing method was adjusted to the colony size. Newly mated *P. lamellidens* queens were reared individually in a plastic tub that was 4.5 cm in length, 2.5 cm in width, and 2.0 cm in height and contained moistened tissue. Each collected ant colony was reared separately in a plastic tub (17.5 cm in length, 8.0 cm in width, 3.0 cm in height, or 20.5 cm in length, 10.5 cm in width, 7.0 cm in height) in which plaster was laid and a feeding area was established. Newly mated *P. lamellidens* queens were fed 5 μl of 50% maple

syrup (Maple Farms Japan, Osaka, Japan) every seven to ten days. Ant colonies were also fed 100–5000 µl of 50% maple syrup every seven to ten days. Worker ants were also fed mealworms, crickets, cockroaches, and/or 8–16 g of Pro Jelly for beetles and stag beetles (Wraios, Saitama, Japan). Newly mated *P. lamellidens* queens were reared in a cool incubator (Mitsubishi Electric Engineering Company, Tokyo, Japan) under dark (0L:24D) conditions at 15–20°C. *C. japonicus* and *C. obscuripes* were reared under light–dark conditions (14L:10D) at 20–30°C. Different rearing conditions were adopted for each species due to my previous rearing experience. In particular, newly mated *P. lamellidens* queens were observed to be sensitive to high temperatures, which is why I adopted the above rearing method.

4-2-2 Induction of rubbing behavior under laboratory conditions

The newly mated *P. lamellidens* queen and host workers (*C. japonicus* or *C. obscuripes*) were placed in plastic cases that contained plaster (5.0 cm × 4.5 cm × 2.5 cm) (Figure 4-1). To prevent possible attacks by the host worker before the newly mated *P. lamellidens* queen performed rubbing behaviors, the host worker was anesthetized through freezing (it was exposed to an environment of 4°C for 2 min and -20°C for 3 min) before the experiment. A newly mated *P. lamellidens* queen could perform the rubbing behavior for three days. When the newly mated *P. lamellidens* queens continued the rubbing behavior for more than one day, I added one new host worker (all from the same colony) every day until the third day. Host workers that spent one week in the nest have used in this experiment. The newly mated *P. lamellidens* queens that were prepared in this process were used for the various experiments that followed. The individuals that performed rubbing behavior at least once after contact with the host worker were used for later experiments. In the isolation rearing, only the host workers were removed from the case, and they were maintained for one to nine days after the rubbing behavior was observed. I conducted the rubbing behavior and isolation rearing experiments under light conditions (24 L:0 D) at 20–30°C and did not feed the ants throughout these experiments. As a control, I also prepared a newly mated *P. lamellidens* queen without exposure to host workers.

4-2-3 Observations of host worker aggressions against the newly mated *P. lamellidens* queen by behavioral tests.

To observe the aggressive behaviors of host workers on the newly mated *P. lamellidens* queen, all host workers were removed immediately after rubbing behavior had occurred for three days, and the newly mated *P. lamellidens* queen was transferred to a plastic cup that was lined with plaster (7.6 cm in diameter and 3.8 cm in height). Three host workers from the same colony as that used in the rubbing behavior experiment were transferred into a plastic cup that contained a newly mated *P. lamellidens* queen that already exhibited rubbing. The first 10 behaviors that host workers performed within 1 min after being transferred to the plastic cup were recorded according to the definitions of my previous study (Iwai et al., 2021). The host workers were selected at random and were used only once in each test. The host workers used for the test were transferred to another case and were not used in subsequent tests.

4-2-4 Application of labeled substances for the tracing assay

I used the stable isotopes of n-triacontane ($C_{30}H_{62}$) and n-dotriacontane ($C_{32}H_{66}$) as labeled substances, and these substances have similar carbon chain lengths to those of the ant CHC (CAS: 93952-07-9, 62369-68-0). Each substance was dissolved individually in hexane (16 mg/mL), 100 µl of the solution was added to a 300 µl micro insert (GLC4010-S630; Shimadzu, Kyoto, Japan), and the solvents were evaporated with nitrogen gas. The solution was stirred with an end-to-end pipette (AS ONE, Osaka, Japan) which are capillary glass tubes, during which nitrogen gas was sprayed to evenly apply the labeled substances over the entire inner wall of the insert. After the solvent was completely volatilized, the labeled substances were applied to the host body surfaces without inducing injury, and this was achieved by placing the host worker into the insert where the labeled substances (n-triacontane-d62: $C_{30}D_{62}$ and n-dotriacontane-d66: $C_{32}D_{66}$) were applied to the inner wall and gently shaking the hosts for 1 h using a rotator.

4-2-5 Extraction and analysis of hydrocarbons

The method for extracting CHCs was modified from a previous study (Akino & Yamaoka, 2012). A single individual was exposed to 4°C for 2 min and then -20°C for 3 min for anesthetic purposes and was then immediately placed in a disposable glass tube (trunk diameter: 10 mm, total height: 75 mm) that contained 200 µl of hexane mixed with saturated alkane (n-docosane: $C_{22}H_{46}$; 10 ng/µl) as an internal standard material. The chemical materials from the body surfaces of ants were extracted by dipping the ants in

hexane for 5 min. After separation in 1X phosphate-buffered saline (PBS), the chemical materials from the postpharyngeal glands (PPG) were extracted by being dipped in hexane and were sonicated using a Bioruptor Ⅱ (BM Equipment, Tokyo, Japan). Next, 0.5 g of silica gel C-200 (Wako Pure Chemical Industries, Tokyo, Japan) was used to fractionate hydrocarbons from the extracted materials. After washing with hexane, silica gel was placed inside a glass pipette that was installed on the stand. Fractionation was carried out by adding the extract inside the pipette. At this time, 1 ml of hexane was added into the pipette to elute hydrocarbons. The obtained hydrocarbons were concentrated by applying nitrogen gas until the solvent hexane was completely evaporated. Thereafter, 50 μl of hexane was added to the vial, and the concentrated CHC was eluted.

The GC-MS system comprised an Agilent 6890N, and an Agilent 5973 MSD was used to measure hydrocarbons. The column used for mass spectrometry (MS) was an HP-5 MS (length 30 m, diameter 0.25 mm, film thickness 0.25 μm; Agilent Technologies, California, USA). 2 μl of sample was injected. The splitless mode was adopted for the sample injection port and the apparatus was maintained at 300°C. Helium was used as the carrier gas at a constant flow rate setting of 0.9 ml/min. The oven temperature was set as follows: 40°C for 3 min, 40 to 260°C at 30°C/min, 260 to 300°C at 15°C/min, and 18 min at the final temperature. C_7 to C_{40} saturated alkanes were used as standard substances.

The estimation of CHCs and statistical analysis were carried out based on peak areas. Equivalent chain length (ECL) of n-alkane, the value of fragment and molecular ions, and the pattern of the mass spectrum was used to estimate each CHC. A previous study (Ozaki et al., 2005) was referred to estimate the CHC of *C. japonicus*. The peak areas were transformed according to $Zij=\ln[Yij/g(Yj)]$, where Yij is the peak area i for the individual j values and $g(Yj)$ is the geometric mean of all peak areas for the individual j values (Reyment, 1989). Hierarchical clustering analysis and correlation analysis were performed using a standardized data matrix of CHC to investigate the similarity between parasite and host CHC profiles. In the hierarchical clustering analysis, the Euclidean distance between each sample was calculated and clustered by the Ward method. In the correlation analysis, the Pearson correlation coefficient was calculated, and the similarity of the CHC profile between each group was evaluated. Statistical analysis was carried out using R software v 4.2.0 (R Core Team, 2022; http://www.R-project.org/), and the gplots package was used to create a heatmap (Warnes et al., 2016; https://cran.r-project.org/web/packages/gplots/). The concentration of hydrocarbons was quantified by

using standards (the mixtures of C_7 to C_{40} saturated alkanes) of known concentrations (0.5, 1, 2, 5, and 10 ng/μl) diluted at five levels, and the internal standard (n-docosane) added in each sample and above standard. After normalizing the peak areas of each standard and sample by using internal standards, a calibration curve was created standard and quantified the concentration of hydrocarbons.

4-2-6 Total RNA extraction

Total RNA extraction was carried out for the whole body (5 larvae, 1 worker, and 1 queen), abdomen (18 newly mated queens), and fat body in the abdomen (17 newly mated queens) of *P. lamellidens* according to a previous study (Kono et al., 2016). Samples were exposed to liquid nitrogen and were placed in a ZR BashingBead lysis tube (Zymo Research, Irvine, CA, United States) that contained 600 μl of TRIzol Reagent (Life Technologies, Carlsbad, CA, United States). The samples were crushed with a Multi-Beads Shocker at 2,500 rpm for 30 s (Yasui Kikai, Osaka, Japan), and total RNA was extracted with a Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA, United States) without DNase treatment. The extraction of total RNA from the abdomen fat body was performed according to Koto et al. (2019). The abdomen fat body was dissected from the newly mated *P. lamellidens* queens in ant saline [4.8 mM Tris-EDTA-SDS (TES), 127 mM sodium chloride (NaCl), 6.7 mM potassium chloride (KCl), 2 mM calcium chloride ($CaCl_2$), and 3.5 mM sucrose] and placed in BioMasher II (Nippi, Tokyo, Japan) that contained 110 μl TRIzol Reagent. After the tissue was disrupted in BioMasher II, extraction was performed with a DNase treatment using a Direct-zol RNA MicroPrep (Zymo Research, Irvine, CA, United States). I performed a quality check (RNA integrity number, quantity, purity ratio) for the total RNA by utilizing TapeStation 2200 RNA Screen Tape (Agilent Technologies, Santa Clara, CA, United States), Qubit Broad Range or High Sensitivity (BR or HS) RNA assay (Life Technologies, Carlsbad, CA, United States), and NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, United States).

4-2-7 Library preparation and complementary DNA (cDNA) sequencing

Illumina sequence libraries were prepared with the extracted total RNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, United States) or KAPA mRNA HyperPrep Kit (KAPA BIOSYSTEMS, Durham, NC, United States). Libraries that contained the whole body and abdomen were prepared using

the NEBNext Ultra RNA Library Prep Kit for Illumina. The messenger RNA (mRNA)s of the whole body and abdomen were isolated by NEBNext Oligo d(T)25 beads (New England BioLabs, Ipswich, MA, United States) from 100–200 ng of total RNA. double-stranded complementary DNA (ds cDNA) was synthesized from the isolated mRNA using ProtoScript II Reverse Transcriptase and NEBNext Second Strand Synthesis Enzyme Mix (New England BioLabs, Ipswich, MA, United States). The synthesized cDNAs were end-repaired by NEBNext End Prep Enzyme Mix and added to NEBNext Adapter for Illumina (New England BioLabs, Ipswich, MA, United States). The addition adapter to cDNA and amplification were achieved through PCR (16 cycles).

The mRNAs of ant fat bodies were isolated by mRNA Capture Beads (KAPA BIOSYSTEMS, Durham, NC, United States) from 100–200 ng of total RNA. ds cDNA was synthesized from the isolated mRNA using KAPA Script and 2nd Strand Synthesis & A-Tailing Enzyme Mix (KAPA BIOSYSTEMS, Durham, NC, United States). The synthesized cDNAs were end-repaired by 2nd Strand Synthesis & A-Tailing Enzyme Mix and Adapter Ligation Master Mix (KAPA BIOSYSTEMS, Durham, NC, United States). The adapter addition to cDNA and amplification were achieved through PCR (16–18 cycles).

The cDNA libraries were sequenced by the NextSeq 500 (Illumina, San Diego, CA, United States) in pairs or single-ends with 150 or 75 cycles of the NextSeq 500/550 High Output Kit v2.0 (Illumina, San Diego, CA, United States). I evaluated the accuracy of sequence reads by FastQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

4-2-8 *de novo* transcriptome assembly and gene prediction

The *de novo* assembly of the transcriptome sequence reads was performed with Trinity v2.8.5 (Grabherr et al., 2011). I performed a quality check of the constructed transcriptome assembly by evaluating BUSCO v2/v3 (reference gene set: Arthropoda) using gVolante v1.2.1 (Nishimura et al., 2017). I used Augustus v3.2.2 to predict the gene regions that were present on the transcriptome assembly using the *Camponotus floridanus* gene model, a closely related species (Stanke & Morgenstern, 2005). CD-HIT-EST (Huang et al., 2010) removed the predicted gene regions with overlapping sequences at the cut-off value of 0.9.

4-2-9 Gene annotation and expression analysis

I annotated CHC synthesis-related genes in the transcriptome assembly of *P. lamellidens* with predicted coding regions. The CHC synthesis-related genes (fatty acid synthase, desaturase, elongase, cytochrome P450 decarbonylase) were annotated using the *Drosophila melanogaster* genes that were registered in UniProt as a query (Q9VQL6_DROME, M9PB21_DROME, ELOF_DROME, Q7K4Y0_DROME, Q9VG68_DROME, A7DZ97_DROME, CP4G1_DROME). After searching for candidate genes in the transcriptome assembly of *P. lamellidens* using BLAST similarity search (E-value < 1E-30; Camacho et al., 2009), the obtained candidate genes and query sequences were subjected to a protein domain search using HMMER v3.1b2 (Eddy, 2011) with the Pfam-A database (E-value ≤ 1E-10). The domain structure was confirmed using DoMosaics v0.95 (Moore et al., 2014). Gene expression was quantified as transcripts per million (TPM) using Kallisto v0.43.0 (Bray et al., 2016). The genes that possessed a domain structure similar to that of *D. melanogaster* and exhibited a TPM value of 10 or higher in any sample were considered to be CHC synthesis-related genes of *P. lamellidens*. I used the R package Edge R v3.18.1 with a false discovery rate (FDR) < 5% for searching differentially expressed gene (DEG) (Robinson et al., 2010). I also annotated DEGs by a tBLASTn similarity search using the nr database.

4-2-10 Reverse transcription-quantitative PCR (RT-qPCR)

I synthesized cDNA from 100 ng of total RNA extracted from the fat body in the abdomens using SuperScript III Reverse Transcriptase (Invitrogen, Waltham, MA, United States). qPCR was performed using a KAPA SYBR Fast qPCR Kit (KAPA BIOSYSTEMS, Durham, NC, United States) and LightCycler 96 (Roche, Basel, Switzerland). The primer list is provided in Table 4-1. Preliminary tests confirmed that the amplification efficiency of all primers was similar (1.8–2.2). I used the housekeeping genes *Gapdh1* and *Actin-5C* as reference genes. The amplicon lengths of all genes were between 143–163 base pair (bp). I calculated the relative expression level of target genes by the E-Method using LightCycler 96 Application software (Roche, Basel, Switzerland).

4-2-11 Protein expression analysis

The fat body from a newly mated queen of *P. lamellidens* was dissected in ant saline. The tissue was crushed in lysis buffer [(12 mM sodium deoxycholate, 12 mM sodium N-

dodecanoylsarcosinate, and 50 mM ammonium bicarbonate containing 1% protease inhibitor cocktail for general use (Nacalai Tesque, Kyoto, Japan)] followed by sonication for 20 min. The lysate (20 µg protein/50 µl) was reacted with 0.5 µl of 1 M dithiothreitol at 37°C for 30 min followed by 2.5 µl of 1 M iodoacetamide at 37°C for 30 min in the dark. After adding 200 µl of 50 mM ammonium bicarbonate, the sample was digested using 0.6 µg of lysyl endopeptidase (Lys-C) (Wako Pure Chemical Industries, Tokyo, Japan) at 37°C for 3 h followed and 0.5 µg of trypsin (Promega, Madison, WI, USA) at 37°C for 16 h. The digest was acidified with trifluoroacetic acid and sonicated for 10 min. The supernatant was desalted using C18-StageTips (Rappsilber et al., 2003) and dried under reduced pressure.

To perform quantitative analysis of key proteins using liquid chromatography-mass spectrometry (LC-MS) analysis, a system equipped with a nanoElute and a timsTOFPro (Bruker Daltonics, Bremen, Germany) was used. The digests that were dissolved in 0.1% formic acid 2% acetonitrile (0.4 µg/µl) were injected into a spray needle column [(ACQUITY UPLC BEH C18, 1.7 µm, ID = 75 µm, length = 25 cm) (Waters Corporation, Milford, MA, USA)] and were separated by gradient analysis. Mixtures of (A) formic acid/water (0.1/100, v/v) and (B) formic acid/acetonitrile (0.1/100, v/v) were used as the mobile phase. The composition of the mobile phase (B) was changed to 2–35% in 100 min and 35–80% in 10 min while maintaining a flow rate of 280 nl/min at 60°C.

Tandem MS was performed using a parallel accumulation serial fragmentation (PASEF) scan mode (Meier et al., 2018). Briefly, the peptides were ionized to positive ion at 1,600 V. Trapped ion mobility spectrometry scanning was performed at a 1/K0 range of 0.7−1.2 Vs/cm^2 with a ramp time of 100 ms while maintaining the duty cycle at 100%. MS scanning was performed at a mass range of *m/z* 300–1,200 followed by 10 PASEF-tandem MS scans per cycle (precursor ion charge = 0–5, intensity threshold = 500, target intensity = 2,000, isolation width = 2 Th at *m/z* 700 and 3 Th at *m/z* 800, collision energy = 20 eV at 0.6 Vs/cm^2, 59 eV at 1.6 Vs/cm^2). The raw data and the associated files were then deposited in the ProteomeXchange Consortium via the jPOST partner repository (accession number: JPST001552) (Okuda et al., 2017).

Protein identification and quantitative analysis of CHC synthesis-related proteins were performed using FragPipe v15.0 containing MSFragger v3.2 and Philosopher v3.4.13 (Kong et al., 2017). The protein sequence database was generated from

transcriptome assembly, and the decoy reversed sequences were added. I also used the cRAP database (https://www.thegpm.org/crap/) to detect contaminant proteins.

The mass tolerance of the precursor and the fragment ions was set to 20 ppm and 0.05 Da, respectively. Mass calibration and parameter optimization were performed using the Philosopher algorithm (da Veiga Leprevost et al., 2020). The enzyme was set to trypsin as a specific cleavage, and up to two missed cleavages were allowed in the proteolysis. The allowed peptide lengths and mass ranges were 7–50 residues and 500–5,000 Da, respectively. Carbamidomethylation was set as a fixed modification at the cysteine residue, whereas N-acetylation at the protein N-term and oxidation at the methionine residue were set as variable modifications, and this allowed for up to three sites per peptide. The peptide spectrum matches and the identified peptides/proteins were determined at < 1% FDR at the protein level.

Quantitative analysis of each peptide was performed with matches between runs (*m/z* tolerance = 20 ppm, retention time tolerance = 3 min, ion mobility tolerance = 0.05 Vs/cm2). After removing outlier values for the identified peptides, the intensities of the identified peptide in the data were normalized (the median $\log_2$ values were unified). The MaxLFQ algorithm (Cox et al., 2014) was used to compare the levels of protein expression between runs using the normalized intensity values of identified unique peptides.

4-2-12 *in situ* hybridization

For the expression analysis of *Cyp4g1* mRNA in the fat body, digoxigenin (DIG)-labeled sense and anti-sense probes (1001 bp) were synthesized by *in vitro* transcription with a DIG RNA Labeling Mix (Roche, Basel, Switzerland). The procedures from the dissection to hybridization were performed as described by Koto et al. (2019). The fat body was dissected from newly mated *P. lamellidens* queens in ant saline and fixed in 4% formaldehyde in phosphate buffer for 20 min at room temperature. The tissues were rehydrated by a decreasing series of methanol (75%, 50%, and 25%) and Tween 20 in PBS [(PTw) (0.1% Tween 20 in 0.1 M PBS)] solutions for 10 min each after being maintained overnight in methanol at -20°C. The rehydrated tissues were digested with 20 μg/ml proteinase K for 3 min in PTw, and then 2 mg/ml glycine in PTw was added twice for 15 min to stop the digestion process. The tissues were post-fixed in PTw with 4% paraformaldehyde (PFA) for 20 min after being rinsed with PTw and then transferred to

50% PTw in a hybridization buffer [50% formamide, 5X saline sodium citrate (SSC), 0.1% Tween 20, 1X Denhardt's solution, 1 mg/ml transfer RNA (tRNA) (Roche, Basel, Switzerland)], 50 μg/ml heparin and 0.1 mg/ml herring sperm DNA (Wako Pure Chemical Industries, Tokyo, Japan) for 10 min. The prehybridization and hybridization processes were carried out by using hybridization buffer and RNA probes. Tissues were hybridized overnight with 100 ng labeled anti-sense or sense RNA probes at 60°C after a prehybridization was performed with hybridization buffer for 2 h at 60°C. When the hybridization process was complete, I transferred tissues into decreasing concentrations of hybridization buffer (75%, 50%, and 25%) in 2X SSC for 30 min each at 60°C to wash and incubated the tissues with 2X SSC and 0.2X SSC for 30 min each. After treatment with DIG buffer I [100 mM tris hydrochloride acid (Tris-HCl) pH 7.5, 150 mM NaCl] for 5 min, the tissues were treated with 1.5% blocking reagent (Roche, Basel, Switzerland) for 1 h to prevent non-specific adsorption of antibody. I performed antigen-antibody reactions using the anti-DIG antibody that was conjugated with alkaline phosphatase (1:1000; Roche, Basel, Switzerland) in DIG buffer I for 1 h. The tissues were washed with DIG buffer I twice for 15 min and then transferred into DIG buffer III [100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM magnesium dichloride ($MgCl_2$), and 0.005% Tween 20] for 3 min. The staining was carried out using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) stock solution (Roche, Basel, Switzerland) for 1 h, and then stop solution [Tris-EDTA (TE) pH 8.0]was applied for 3 min. Finally, the stained tissues were washed with DIG buffer III twice for 10 min and PTw for 5 min. The tissues were mounted with 70% glycerol. The images of mounted tissues were acquired by the VHX-5000 system (Keyence, Osaka, Japan).

4-2-13 Data Availability Statement

The data of transcriptome analysis presented in this study are deposited in the NCBI repository, accession number PRJNA821370 (BioProject). The data of LC-MS analysis presented in the study are deposited in the JPOST repository, accession number JPST001552. The transcriptome assembly presented in the study are deposited in the NCBI repository, accession number GJVV01000000 (the sequences less than 200 bp in registered assembly were removed).

Table 4-1. The list of primers for RT-qPCR.

Gene name	Primer type	Sequence (5' to 3')
Gapdh1 (g57081.t1)	Forward	CGTATTGGCCGTCTTGTACTG
	Reverse	CGGCTTTGACTTCTCCCTTG
Actin5C (g5939.t1)	Forward	TCGCGACATCAAGGAAAAGC
	Reverse	ACAGCGGAATCGTTCGTTAC
Desat (g34819.t1)	Forward	GGAAGTCAAGGAAAAGGGCAAG
	Reverse	TCCAACACACAACCGGAATG
Cyp4g1 (g28547.t1)	Forward	TGCGCCTTAGGAAATCATCAG
	Reverse	ACGGTACTGGTGGGAAAAGTC

4-3 Results

4-3-1 Induction of rubbing behavior under laboratory conditions

To perform quantitative assessments, first, the rubbing behavior of a newly mated *P. lamellidens* queen (the new queen) was re-enacted in a laboratory environment (Figure 4-1). The rubbing behavior experiment was carried out in a plaster-lined plastic case (5.0 × 4.5 × 2.5 cm), and the new queen interacted with the host workers in the case. To reduce the risk of counterattack from the host workers, the host workers were anaesthetized before being placed with the new queen (the detailed procedures are described in the Materials and Methods section of Chapter 4). The rubbing behavior observed in the field is characterized by the queen straddling the host worker and rubbing it with her legs (Figure 4-1A; Kohriba, 1963 & 1966; Kubota, 1974; Sakai, 1990, 1996, & 2000; Yano, 1911), and I succeeded in observing the same behavior in the laboratory environment. In addition, this rubbing behavior of the new queen was similar for both known host workers, *C. japonicus* (Japanese Ant Database Group, 2003; Kohriba, 1963) and *C. obscuripes* (Iwai et al., 2021) (Figures 4-1BC).

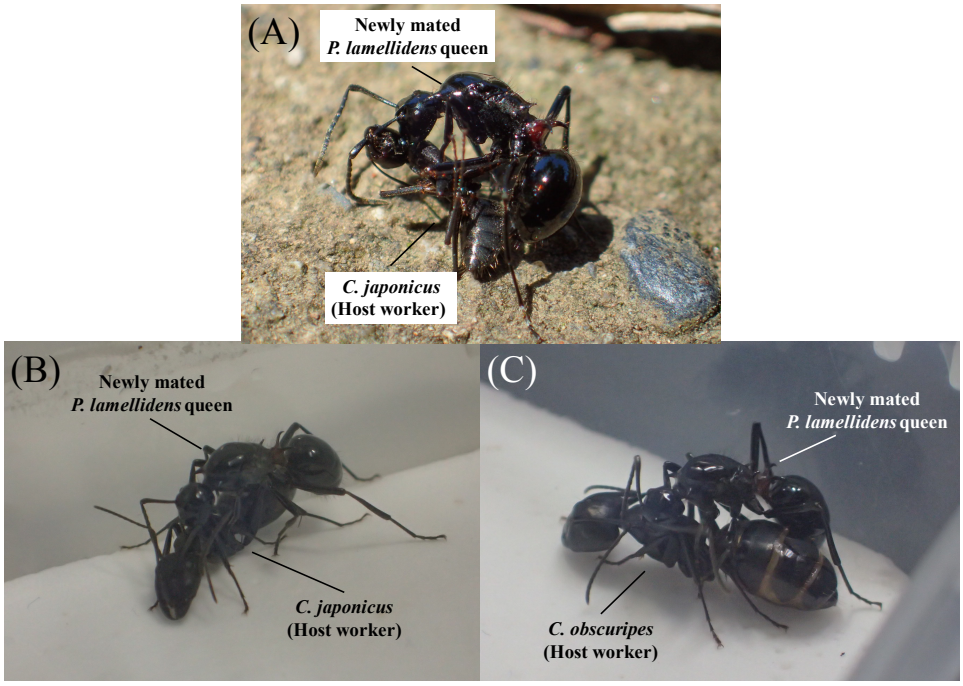


Figure 4-1. The rubbing behavior of newly mated *P. lamellidens* queens. (A) The newly mated *P. lamellidens* queen exhibits rubbing behavior towards its host worker (*C. japonicus*) in a natural environment in a mixed forest in Nagaoka City, Niigata Prefecture (N 37°25′51″, E 138°52′53″). (B) Rubbing behavior against *C. japonicus* workers under laboratory conditions. (C) Rubbing behavior against *C. obscuripes* workers under laboratory conditions.

4-3-2 The effect of rubbing behaviors on host workers

A behavioral test was performed to examine how rubbing behaviors changed the nestmate discrimination displayed by host workers. The behavioral test measured the actions of host workers towards the new *P. lamellidens* queen after three days of rubbing behavior was observed on the host worker. At this time, the rubbing behavior was performed on the worker from the same colony as that of the workers used for the behavioral test. When the newly mated *P. lamellidens* did not perform rubbing behaviors, *C. japonicus* workers recognized *P. lamellidens* as a non-nestmate and a high frequency of aggressive behavior was observed (Figure 4-2A). On the other hand, *C. japonicus* workers did not attack the new queen that had exhibited rubbing behavior (Figure 4-2A). This finding suggests that the rubbing behavior enabled the newly mated queen of *P. lamellidens* to deceive the system of host nestmate recognition.

To determine how the rubbing behavior altered the response of the host workers, GC-MS analysis was conducted. The mass spectrometer measured the CHCs on the body surface of new queens that had or had not exhibited the rubbing behavior, as well as the host workers that were used as the rubbing target. As a result, the CHC profile of the newly mated *P. lamellidens* queens that had exhibited rubbing behavior had clearly changed to one that closely resembled the host worker profile (Figures 4-2BC and 4-3, Table 4-2, $R > 0.7$, p-value < 0.01). Interestingly, the total amount of CHCs of the new queen increased due to the rubbing behavior (Figure 4-2D). The profiles were also confirmed to change even when the target of rubbing behavior was *C. obscuripes* (Figures 4-4 and 4-5, Table 4-3). These results suggest that the new queen can disguise her CHC profile and amount of CHCs by rubbing regardless of the host species to avoid host worker aggression.

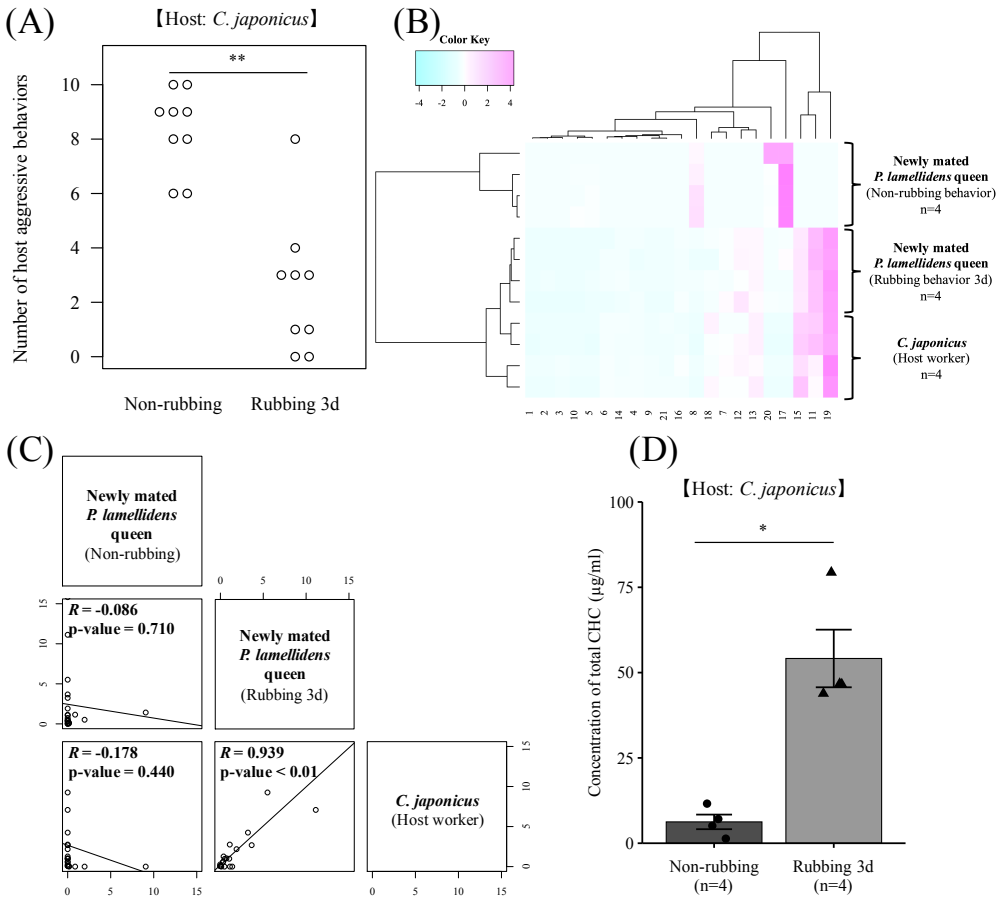


Figure 4-2. The CHC profile and effect of rubbing behavior on the host worker (*C. japonicus*) reaction. I used *C. japonicus* as a host worker in these experiments. (A) The number of aggressive behaviors of host workers against newly mated *P. lamellidens* queens. These host workers were prepared with nine biological replicates, and they belonged to the same colony as that used in the rubbing behavior experiments but with different individuals. Ten actions were observed against the newly mated *P. lamellidens* queen, and among these actions, “threaten”, “rush”, and “bite” were used to count the number of host aggressions. Wilcoxon signed rank test, **; significant difference: p-value < 0.01 (the actual value: 0.003). (B) Hierarchical clustering analysis of CHC profiles of newly mated *P. lamellidens* queens and host workers. This analysis was performed using the standardized value of the peak area that was converted to a Z score. The x-axis indicates the type of estimated hydrocarbons (see Table 4-2). (C) Correlation analysis of CHC profiles of newly mated *P. lamellidens* queens and host workers. *R* indicates the Pearson correlation coefficient. Each plot shows the detected CHCs. The x-axis and the y-axis show the area values of the standardized average peak area value. (D) The concentration of CHC in newly mated *P. lamellidens* queens. Mann–Whitney U test, *; significant difference: p-value < 0.05 (the actual value: 0.028), n=4. Error bar indicates standard error. Three days is refered to as “3d”.

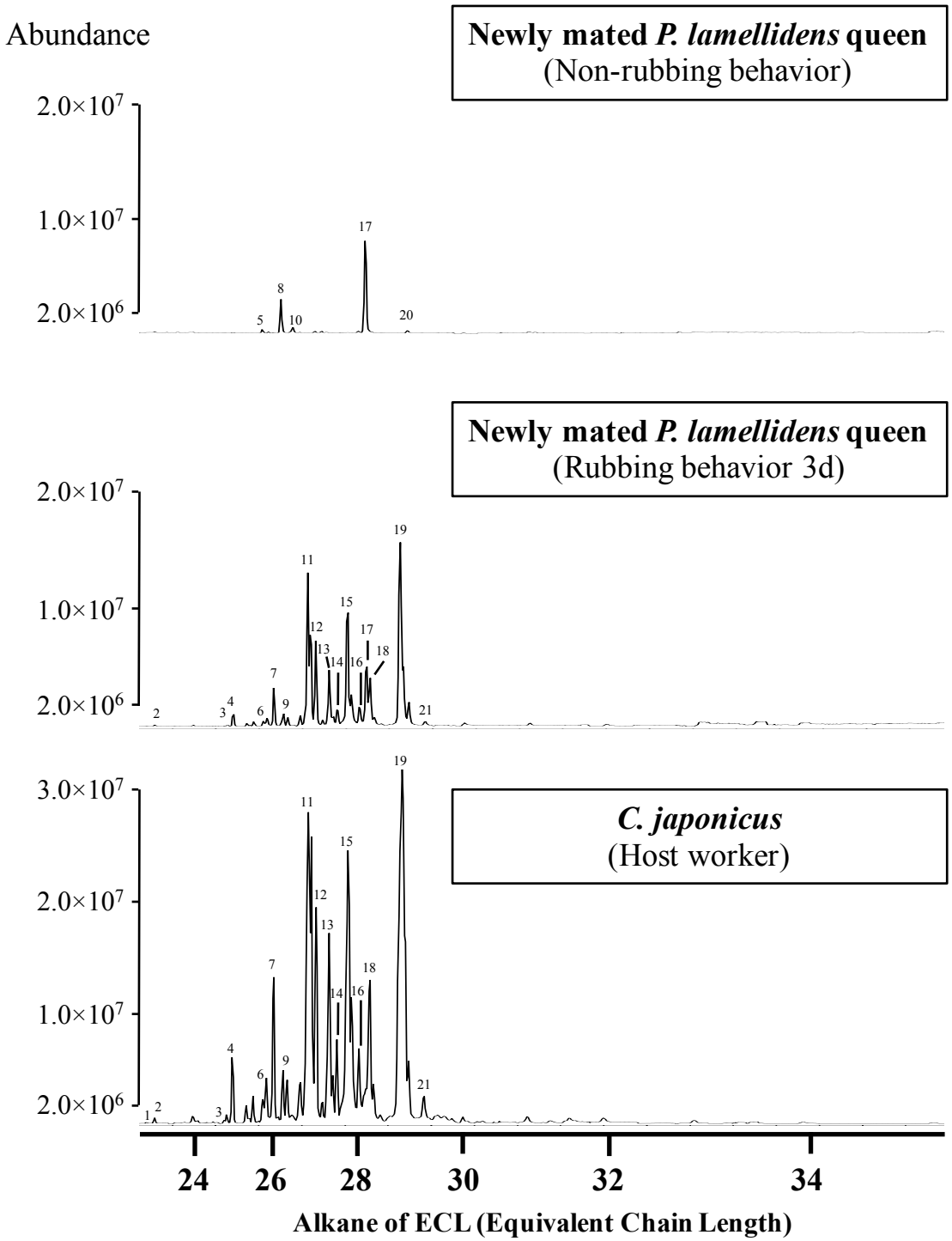


Figure 4-3. Examples of CHC chromatograms of newly mated *P. lamellidens* queens and their host (*C. japonicus*). The number assigned to each peak indicates the type of estimated hydrocarbon (see Table 4-2). Three days is refered to as "3d".

Table 4-2. The list of estimated CHCs from newly mated *P. lamellidens* queens and *C. japonicus*.

Peak no.	ECL of n-alkane	CHC	*P. lamellidens* queen Non-rubbing behavior	*P. lamellidens* queen (Isolated after rubbing behavior)	*C. japonicus* worker
1		$C_{23:1}$	-	+	+++
2	23	nC_{23}	-	+++	+++
3		$C_{25:1}$	-	++	+++
4	25	nC_{25}	++	+++	+++
5		7,12-dimeC_{25}	++	++	-
6		$C_{26:1}$	-	+++	+++
7	26	nC_{26}	++	+++	+++
8		5,10-dimeC_{26}	++	+	-
9		5,7,12- and 7,9,12-trimeC_{25}	-	+++	+++
10		Unknown alkane	+	++	-
11		$C_{27:1}$	-	+++	+++
12	27	nC_{27}	++	++	+++
13		13-meC_{27}	-	+++	+++
14		5-meC_{27}	-	+++	+++
15		7,15-dimeC_{27}	-	+++	+++
16	28	nC_{28}	++	++	+++
17		5,10,15- trimeC_{27}	++	++	-
18		5,7,12-trimeC_{27}	-	++	+++
19		$C_{29:1}$	-	+++	+++
20	29	nC_{29}	++	++	-
21		13-meC_{29}	-	+++	+++

+++/- indicates CHCs that were commonly detected in all ant samples or not detected at all. CHCs detected in more or less than half of ant samples are represented by ++ or +. ECL of n-alkane: equivalent chain length of n-alkane. The number of CHCs was counted from ant samples which *C. japonicus* was used in the experiment.

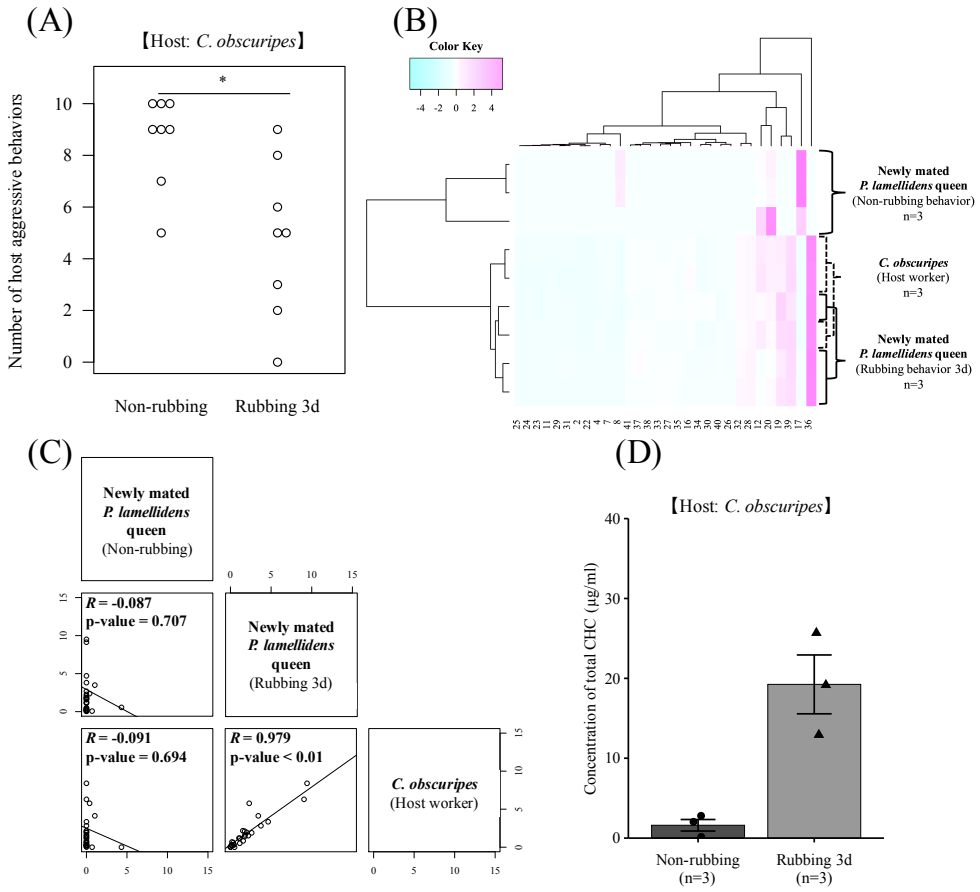


Figure 4-4. The CHC profile for the effect of rubbing behavior on the host worker (*C. obscuripes*) reaction. I used *C. obscuripes* as a host worker in these experiments. (A) The number of incidences of aggressive behavior by host workers towards newly mated *P. lamellidens* queens. These host workers were prepared with eight biological replicates, and they belonged to the same colony that was used in the rubbing behavior experiments but the individual host workers were different. Ten actions by the host workers against the newly mated *P. lamellidens* queen were observed, and among these actions, "threaten", "rush", and "bite" were used to count the incidences of host aggression towards the queens. Wilcoxon signed rank test, *; significant difference: p-value < 0.05 (the actual value: 0.023). (B) Hierarchical clustering analysis of the CHC profiles of newly mated *P. lamellidens* queens and host workers. This analysis was performed using the standardized value of peak area, which was converted to the Z score. The x-axis indicates the type of estimated hydrocarbons (see Table 4-3). (C) Correlation analysis of the CHC profiles of newly mated *P. lamellidens* queens and host workers. *R* indicates the Pearson correlation coefficient. Each plot shows the CHCs that were detected. The x-axis and the y-axis show the area values of the standardized average peak area value. (D) The concentration of CHC in newly mated *P. lamellidens* queens. n=3. Error bar indicates standard error. Three days is refered to as "3d".

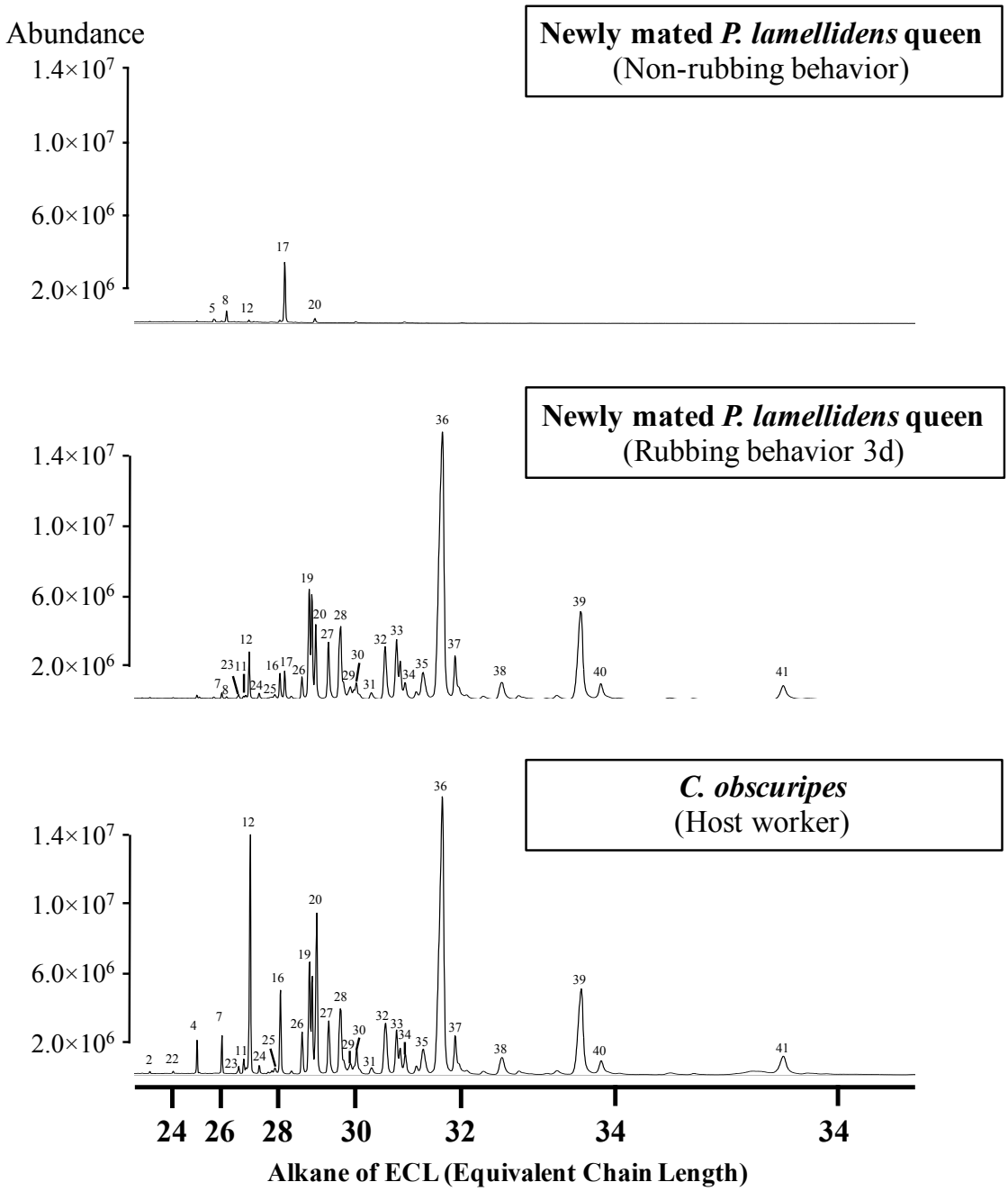


Figure 4-5. Examples of the CHC chromatograms of newly mated *P. lamellidens* queens and their hosts (*C. obscuripes*). The number assigned to each peak indicates the type of estimated hydrocarbon (see Table 4-3). Three days is refered to as "3d".

Table 4-3. The list of estimated CHCs from newly mated *P. lamellidens* queens and *C. obscuripes*.

Peak no.	ECL of n-alkane	CHC	*P. lamellidens* queen Non-rubbing behavior	*P. lamellidens* queen (Isolated after rubbing behavior	*C. obscuripes* worker
2	23	nC_{23}	-	++	+++
22	24	nC_{24}	-	++	+++
4	25	nC_{25}	-	+++	+++
7	26	nC_{26}	-	+++	+++
8		5,10-dimeC_{26}	+	+++	-
23		2-meC_{26}	-	+++	+++
11		$C_{27:1}$	-	+++	+++
12	27	nC_{27}	+++	+++	+++
24		11-meC_{27}	-	+++	+++
25		$C_{28:1}$	-	+++	+++
16	28	nC_{28}	-	+++	+++
17		5,10,15- trimeC_{27}	+++	+++	-
26		3-meC_{28}	-	+++	+++
19		$C_{29:1}$	-	+++	+++
20	29	nC_{29}	+++	+++	+++
27		Unknown alkane	-	+++	+++
28		9,13-dimeC_{29}	-	+++	+++
29		$C_{30:1}$	-	+++	+++
30	30	nC_{30}	-	+++	+++
31		12-meC_{30}	-	+++	+++
32		Unknown alkane	-	+++	+++
33		$C_{31:1}$	-	+++	+++
34	31	nC_{31}	-	+++	+++
35		Unknown alkane	-	+++	+++
36		11,16-dimeC_{31}	-	+++	+++
37		Unknown alkane	-	+++	+++
38		10,16-dimeC_{32}	-	+++	+++
39		11,17-dimeC_{33}	-	+++	+++
40		Unknown alkane	-	+++	+++
41		Unknown alkane	-	+++	+++

+++/- indicates CHCs that were commonly detected in all ant samples or not detected at all. CHCs detected in more or less than half of ant samples are represented by ++ or +. ECL of n-alkane: equivalent chain length of n-alkane. The number of CHCs was counted from ant samples which *C. obscuripes* was used in the experiment.

4-3-3 Tracing assay with labeled CHCs

MS and behavioral tests demonstrated that the rubbing behavior enables the host CHC profile to be disguised. However, whether this chemical disguise is implemented by chemical camouflage or chemical mimicry is unclear. Here, I focused on the result that the total amount of CHCs in the new queen was increased by the rubbing behavior (Figure 4-2D). Therefore, whether this chemical disguise is implemented by direct deprivation from the body surface of the host worker (chemical camouflage) was verified by a tracing assay in which substances labeled with stable isotopes were applied. The tracking experiments were conducted using two types of hydrocarbons ($C_{30}D_{62}$ and $C_{32}D_{66}$), in which the hydrogens of hydrocarbons with a chain length similar to those possessed by the host worker (*C. japonicus*; Table 4-2) were replaced by stable isotopes. The efficiency of applying labeled substances to the ant body was confirmed with GC-MS measurements, and the labeled substances accounted for approximately 10–20% of the total CHC in the host worker (Figure 4-6A). A similar ratio was also confirmed in the newly mated *P. lamellidens* queens, which exhibited rubbing behaviors (Figure 4-6B). A new queen could perform rubbing behavior on the labeled host worker for three days. By rubbing, the newly mated *P. lamellidens* queens acquired both the host CHC and the labeled substances on their cuticle from these host workers (Figures 4-7 and 4-8A–C). In addition, since PPGs are involved in the storage of hydrocarbons in ants (Soroker et al., 1994 & 1995), I analyzed the hydrocarbon content in the PPG of the newly mated *P. lamellidens* queens. Host CHC and labeled substances were also detected in the PPG of newly mated *P. lamellidens* queens (Figures 4-8D and 4-9). These results suggest that the new queen directly obtains the host CHC through rubbing behavior.

To observe how long the new queen can retain the acquired CHC, including the labeled substances, the new queens were isolated for up to nine days after rubbing behavior was observed against the labeled host worker, and the amount of CHC and CHC profile were measured. The newly mated *P. lamellidens* queen maintained the disguised CHC profile for at least nine days of isolation (Figures 4-8E and 4-10AB). Furthermore, the quantity of disguised CHC and labeled substances did not significantly increase or decrease during the nine days (Figures 4-8A–C and 4-10C).

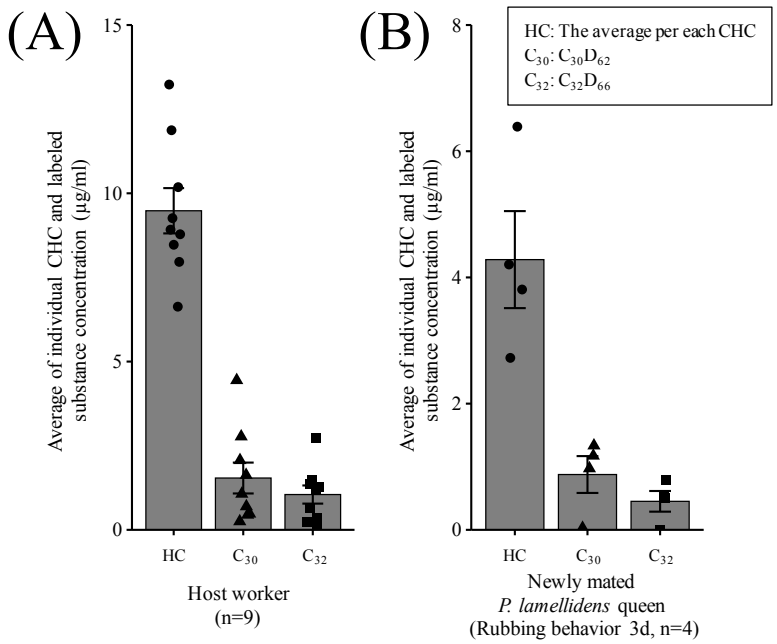


Figure 4-6. The concentration of CHC and labeled substances in newly mated *P. lamellidens* queens and host workers. The concentration of labeled substances ($C_{30}D_{62}$ and $C_{32}D_{66}$) and the average concentration per CHC of the host workers (*C. japonicus*) (A) and newly mated *P. lamellidens* queens at three days of the rubbing behavior (Rubbing behavior 3d) (B). The host workers remained separated from the newly mated *P. lamellidens* queens for one to three days after the labeled substances were applied. For three days, the newly mated *P. lamellidens* queens exhibited rubbing behaviors towards a labeled host worker. All error bars indicate the standard error.

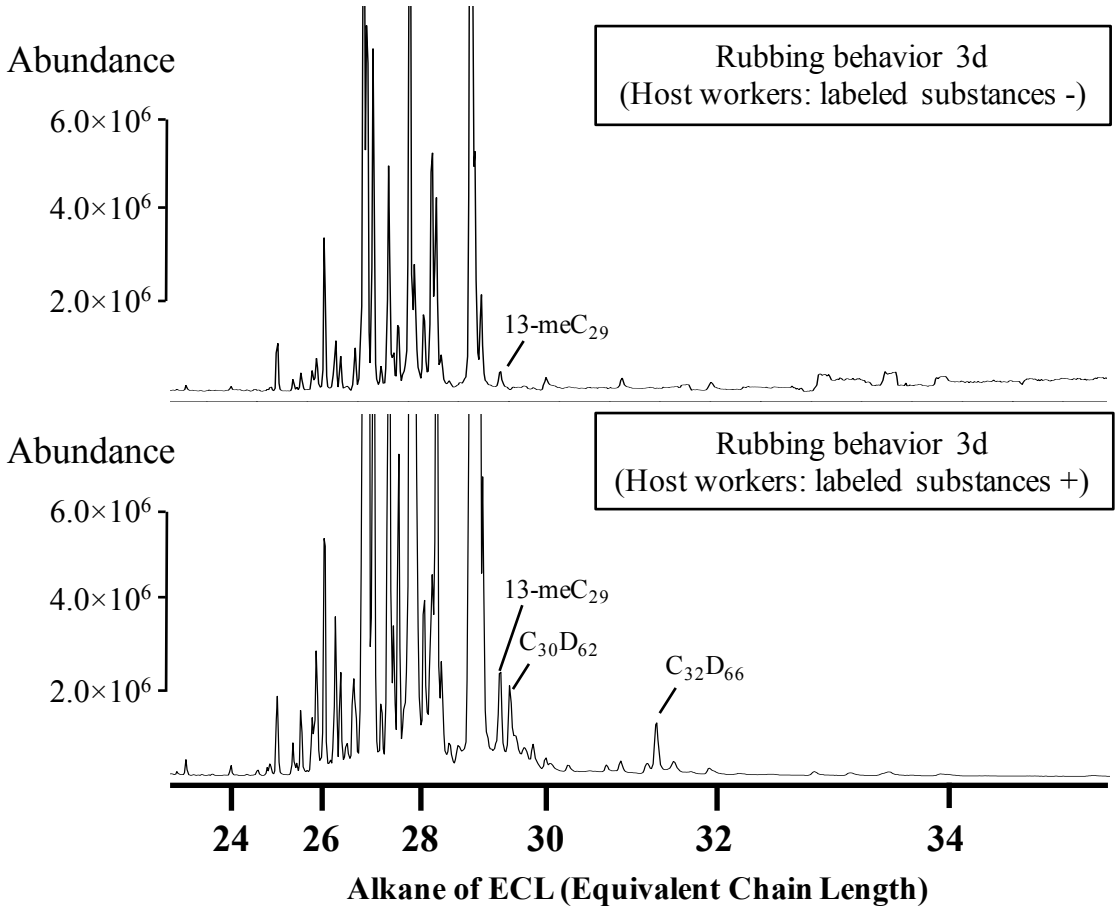


Figure 4-7. The chromatogram of labeled substances ($C_{30}D_{62}$ and $C_{32}D_{66}$) from the cuticle of a newly mated *P. lamellidens* queen. The upper panel shows the chromatograms of the newly mated *P. lamellidens* queens that exhibited rubbing behaviors towards the host workers at three days (Rubbing behavior 3d) without labeled substances, and the lower panel shows the chromatograms of newly mated *P. lamellidens* queens that performed rubbing behaviors towards the host workers at three days (Rubbing behavior 3d) with labeled substances.

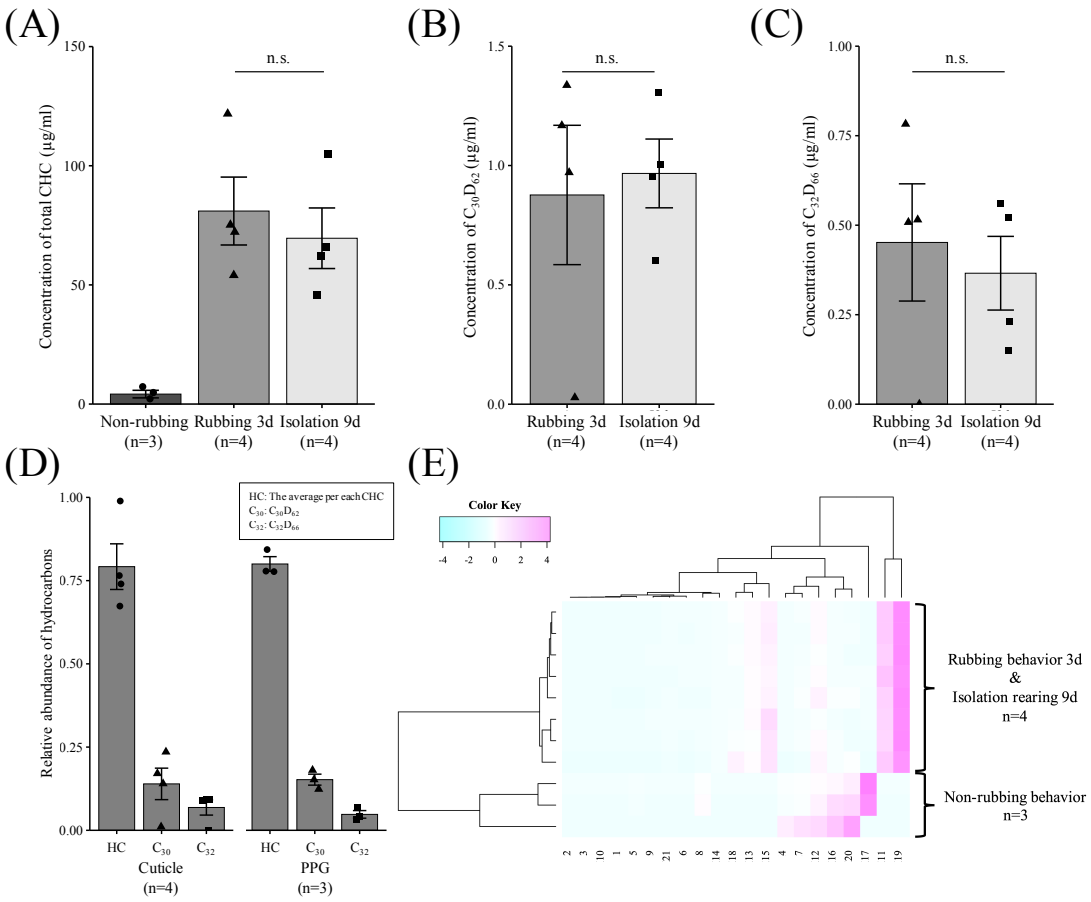


Figure 4-8. Comparison of CHC and labeled substances in the newly mated *P. lamellidens* queens. These experiments used *C. japonicus* as a host worker (A–E). (A) The concentration of CHC in the newly mated *P. lamellidens* queens at three days of the rubbing behavior (Rubbing 3d) and at nine days of isolation (Isolation 9d). [Mann–Whitney U test, n.s.; nonsignificant difference (the actual value: 0.485), n=4]. (B&C) The concentration of labeled substances ($C_{30}D_{62}$ and $C_{32}D_{66}$) in newly mated *P. lamellidens* queens (Mann–Whitney U test, n.s.; non-significant difference (the actual value: 0.885–1.000), n=4). (D) The ratio of hydrocarbons (the average per CHC, $C_{30}D_{62}$, and $C_{32}D_{66}$) on the newly mated *P. lamellidens* queen cuticle and PPGs after rubbing behavior. The average ratios of hydrocarbons identified in the cuticle and PPGs were correlated (Pearson method, $R = 1$, p-value < 0.01 (the actual value: 0.000)). (E) Hierarchical clustering analysis of the CHCs of newly mated *P. lamellidens* queens at three days of the rubbing behavior (Rubbing behavior 3d) and at nine days of isolation (Isolation rearing 9d). This analysis was performed using the peak area value that was converted to a Z score. The number on the x-axis indicates the type of estimated hydrocarbon (see Table 4-2). All error bars indicate the standard error.

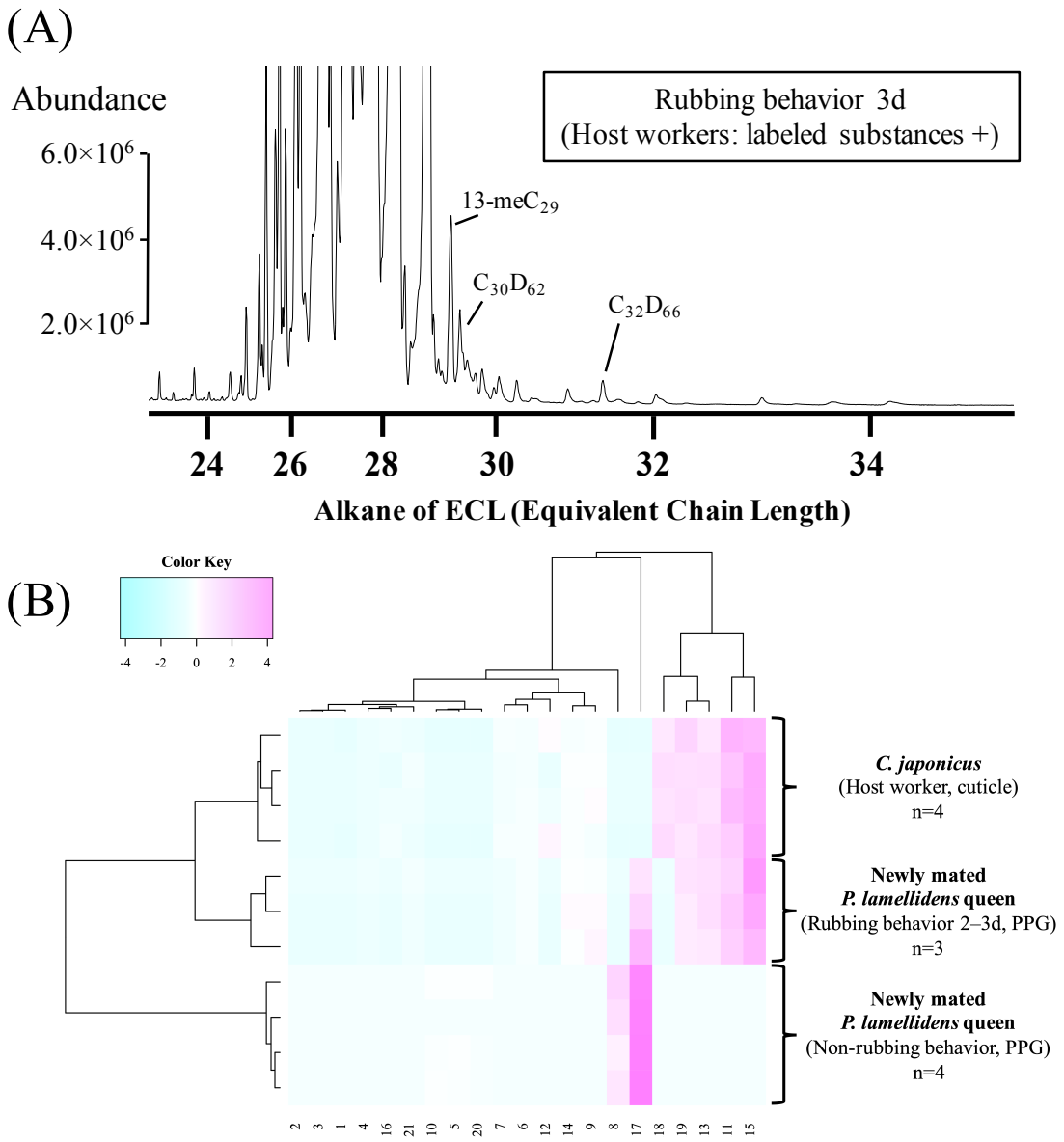


Figure 4-9. The hydrocarbons in the PPGs of newly mated *P. lamellidens* queens. (A) The chromatogram of labeled substances ($C_{30}D_{62}$ and $C_{32}D_{66}$) in the PPGs of newly mated *P. lamellidens* queens at three days of the rubbing behavior (Rubbing behavior 3d). This ant exhibited rubbing behaviors towards the host workers with labeled substances. (B) Hierarchical clustering analysis of the hydrocarbons in the PPGs and cuticle of a newly mated *P. lamellidens* queen at two to three days of the rubbing behavior (Rubbing behavior 2–3d) and host workers (*C. japonicus*). This analysis was performed using the peak area value that was converted to the Z score. The number on the x-axis indicates the type of estimated hydrocarbon (see Table 4-2). One of the new queens died on the second day while performing the rubbing behavior, at which point hydrocarbons were extracted.

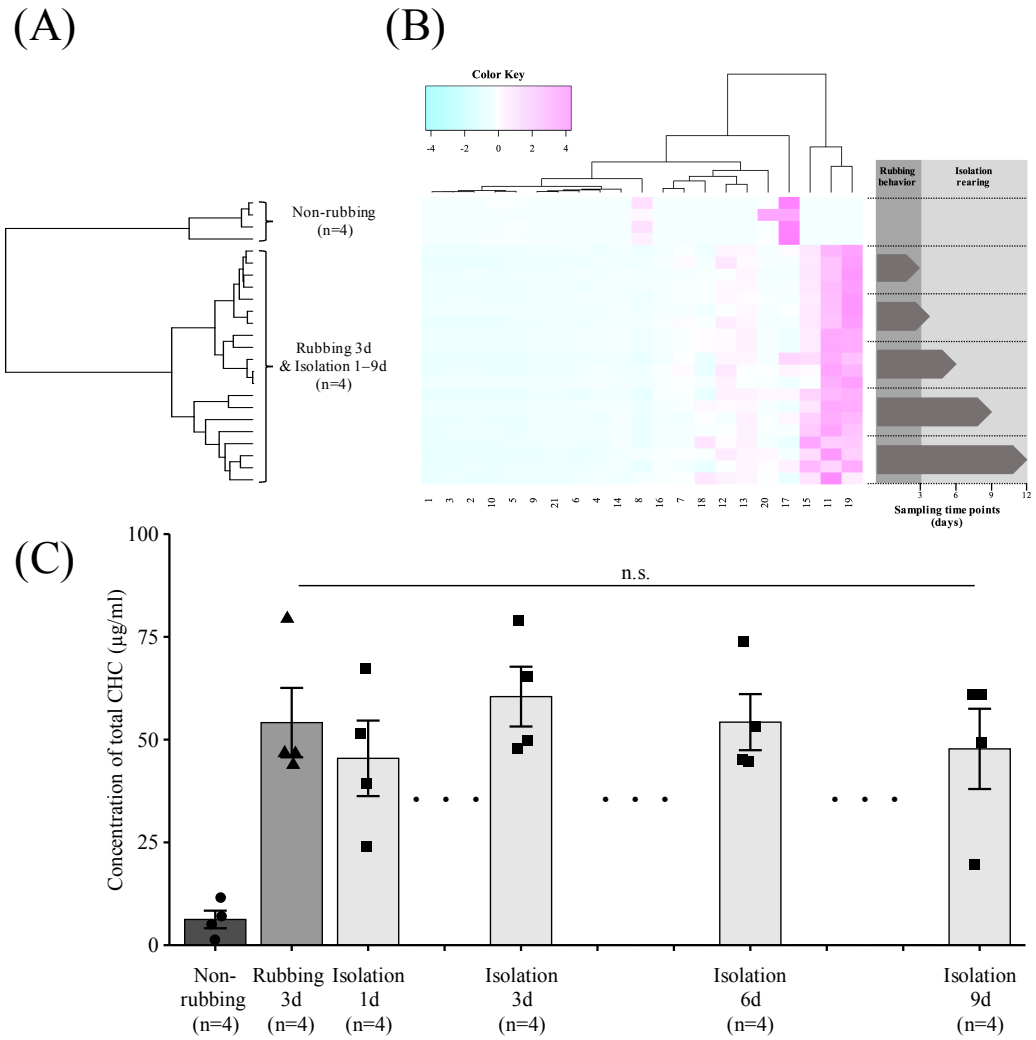


Figure 4-10. Transition of the CHC in newly mated *P. lamellidens* queens during isolation rearing. (A) Hierarchical clustering analysis of CHC in the newly mated *P. lamellidens* queen at three days of the rubbing behavior (Rubbing 3d) and at one, three, six, and nine days of isolation (Isolation 1–9d). This analysis was performed using the peak area value that was converted to the Z score. Newly mated *P. lamellidens* queens exhibited rubbing behavior (three days) against their hosts before being isolated from these hosts. (B) Heatmap of CHC in the newly mated *P. lamellidens* queens. This analysis was also performed using the peak area value that was converted to the Z score and used newly mated *P. lamellidens* queens that exhibited rubbing behavior (three days) against their hosts before they were isolated from these hosts. The number on the x-axis indicates the type of estimated hydrocarbon (see Table 4-2). (C) The concentration of CHC in the newly mated *P. lamellidens* queens at three days of the rubbing behavior (Rubbing 3d) and at one, three, six, and nine days of isolation (1d, 3d, 6d, and 9d). [Mann–Whitney U test, n.s.; nonsignificant difference (the actual value: 0.885), n=4]. The error bars indicate the standard error.

4-3-4 Gene and protein expression profiling

The tracing assay with labeled CHC suggested that the chemical disguise of *P. lamellidens* may be based on a "chemical camouflage" method. I next examined another disguising strategy, "chemical mimicry". Since chemical mimicry is a biosynthesis-mediated strategy, expression profiling was performed. A reference transcriptome of *P. lamellidens* was established by extracting the total RNA from the whole body of the larvae, worker, queen, abdomen of the newly mated queen, and fat body of the newly mated queen. cDNA was synthesized from the obtained total RNA and sequenced by an Illumina sequencer. Paired-end and single-end sequencing of 75–150 bp produced a total of 210 M reads (Table 4-4). I assembled these reads using Trinity v2.8.5 and constructed the reference assembly of 23,523 coding regions with a BUSCO completeness of 96.6% (Table 4-5).

The expression levels of the newly mated queen were analyzed by RNA-seq with the *P. lamellidens* reference assembly, and these analyzes were performed for queens without rubbing behavior (non-rubbing behavior) and queens with rubbing behavior to the host workers for three days (rubbing 3d), or queens that were isolated from the hosts for nine days after rubbing behavior was observed (isolation 9d). There are five CHC synthesis-related genes (Figure 4-11A), and these were expressed on the oenocytes that are contained in the abdomen fat body (Koto et al., 2019; Martins & Ramalho-Ortigao, 2012; Roma et al., 2006 & 2008; Thiele & Camargo-Mathias, 2003). Fatty acid synthase is involved in the biosynthesis of CHC precursors, desaturase and elongase in the construction of CHC varieties (it is involved in extending the chain length and inserting double bonds), and fatty acyl-CoA reductase and cytochrome P450 decarbonylase are involved in the final modification of CHC. The expression level of most of the genes that are related to CHC synthesis did not change among the non-rubbing, rubbing 3d, and isolation 9d groups, except for desaturase (*Desat*) (Figure 4-11, Tables 4-6, 4-7, and 4-8). Desaturase (*Desat*) (g34819.t1), a CHC synthesis-related gene, was changed at the mRNA level but not at the protein level (Figures 4-11BC and 4-12AB, Tables 4-6 and 4-7). The expression level of cytochrome P450 decarbonylase (*Cyp4g1*), which is specifically expressed in the oenocyte (Koto et al., 2019), is particularly known to directly correlate with the amount of synthesized CHC. In this study, cytochrome P450 decarbonylase (*Cyp4g1*) (g28547.t1) was not detected as a DEG by transcriptome analysis (Figures 4-11DE, 4-12CD, and 4-13, Tables 4-7 and 4-8). In addition, the

expression pattern of cytochrome P450 decarbonylase (*Cyp4g1*) was examined by RT-qPCR analysis (Figure 4-12D) and a quantitative analysis of key proteins using LC-MS analysis (Figure 4-11E). These analyses also demonstrated that the expression level of *Cyp4g1* was not changed significantly in mRNA and protein level.

Table 4-4. The list of RNA-Seq data from *P. lamellidens* obtained in this work.

Sample type	Body part	Description	Sequence type	Accession No.	
Larva	Whole body	The pool of five larvae	Paired-end	SRR18532324	*
Worker	Whole body		Paired-end	SRR18532323	*
Queen	Whole body		Paired-end	SRR18532312	*
Newly mated queen	Abdomen	No rubbing behavior	Single-end	SRR18532303	*
Newly mated queen	Abdomen	No rubbing behavior	Single-end	SRR18532302	*
Newly mated queen	Abdomen	No rubbing behavior	Single-end	SRR18532301	*
Newly mated queen	Abdomen	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532300	*
Newly mated queen	Abdomen	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532299	*
Newly mated queen	Abdomen	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532298	*
Newly mated queen	Abdomen	Rubbing to the *C. obscuripes* workers for three days	Single-end	SRR18532297	*
Newly mated queen	Abdomen	Rubbing to the *C. obscuripes* workers for three days	Single-end	SRR18532322	*
Newly mated queen	Abdomen	Rubbing to the *C. obscuripes* workers for three days	Single-end	SRR18532321	*
Newly mated queen	Abdomen	Isolated for nine days after the rubbing behavior to the *C. japonicus* workers	Single-end	SRR18532320	*
Newly mated queen	Abdomen	Isolated for nine days after the rubbing behavior to the *C. japonicus* workers	Single-end	SRR18532319	*
Newly mated queen	Abdomen	Isolated for nine days after the rubbing behavior to the *C. japonicus* workers	Single-end	SRR18532318	*
Newly mated queen	Abdomen	Isolated for nine days after the rubbing behavior to the *C. obscuripes* workers	Single-end	SRR18532317	*
Newly mated queen	Abdomen	Isolated for nine days after the rubbing behavior to the *C. obscuripes* workers	Single-end	SRR18532316	*
Newly mated queen	Abdomen	Isolated for nine days after the rubbing behavior to the *C. obscuripes* workers	Single-end	SRR18532315	*
Newly mated queen	Fat body	No rubbing behavior	Single-end	SRR18532314	
Newly mated queen	Fat body	No rubbing behavior	Single-end	SRR18532313	
Newly mated queen	Fat body	No rubbing behavior	Single-end	SRR18532311	
Newly mated queen	Fat body	No rubbing behavior	Single-end	SRR18532310	
Newly mated queen	Fat body	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532309	
Newly mated queen	Fat body	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532308	
Newly mated queen	Fat body	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532307	
Newly mated queen	Fat body	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532306	
Newly mated queen	Fat body	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532305	
Newly mated queen	Fat body	Rubbing to the *C. japonicus* workers for three days	Single-end	SRR18532304	

The BioProject ID of these data is PRJNA821370. *Each data was used to transcriptome assembly.

Table 4-5. Assembly statistics of the *P. lamellidens* transcriptome.

	P. lamellidens
Number of predicted genes	23,523
Average scaffold length	1,187 bp
Total scaffold length	27,914,451 bp
Longest scaffold	40,884 bp
Scaffold N50	2,187 bp
BUSCO (Arthropoda)	96.6%

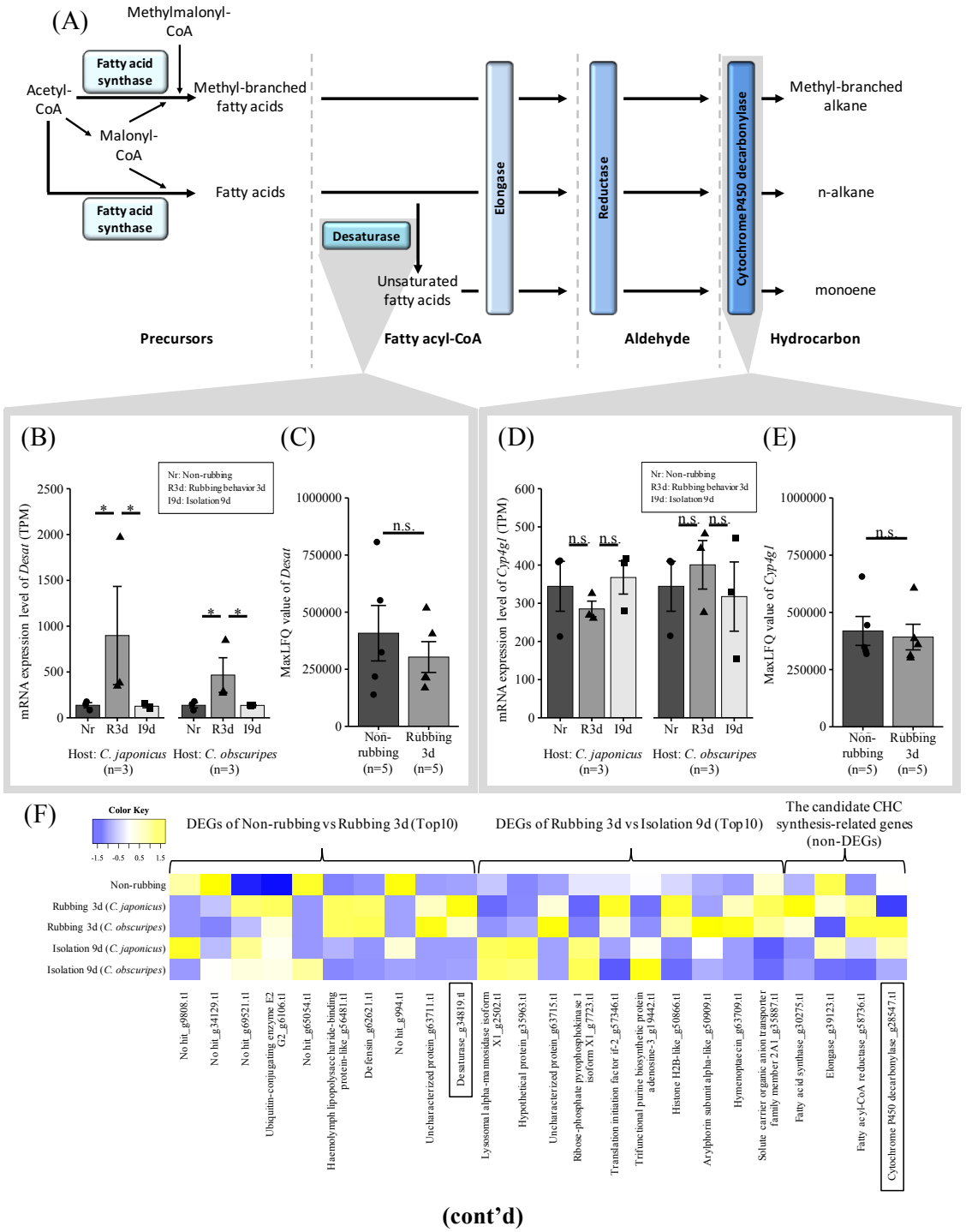
Table 4-6. The number of DEGs in the abdomen.

	C. japonicus	***C. obscuripes***	**Common in both**
Non-rubbing vs. Rubbing 3d	65	103	20
Rubbing 3d vs. Isolation 9d	807	247	74

Significant difference: FDR < 0.05

Table 4-7. The candidate CHC synthesis-related genes in transcriptome assembly.

Gene name	UniProt ID	Gene ID	Identity of BLAST (%)
Fatty acid synthase (*FASN2*)	Q9VQL6_DROME, M9PB21_DROME	g30275.t1	49.461−49.482
Elongase (*eloF*)	ELOF_DROME	g39123.t1, g56534.t1, g36733.t1, g21655.t1, g23416.t1, g23415.t1, g24854.t1	34.000−38.554
Desaturase (*Desat1, Desat2, desatF*)	Q7K4Y0_DROME, Q9VG68_DROME, A7DZ97_DROME	g34819.t1, g11236.t1, g11238.t1, g53824.t1, g49648.t1, g53826.t1	37.829−69.006
Fatty acyl-CoA reductase (*Sgp*, *anon-WO0140519.58*, *anon-WO0140519.58*, *Dmel＼CG4020*)	Q9VLJ7_DROME, Q9VG86_DROME, Q9VES6_DROME, Q9W459_DROME	g58736.t1, g43597.t1, g45448.t1, g45454.t1, g61926.t1, g24041.t1, g56854.t1, g56866.t1, g22537.t1, g24040.t1, g31109.t1, g24045.t1, g50718.t1, g50719.t1, g15289.t1, g18683.t1	27.105−41.628
Cytochrome P450 decarbonylase (*Cyp4g1*)	CP4G1_DROME	g28547.t1, g28546.t1, g57012.t1, g20324.t1, g12257.t1, g37042.t1, g20315.t1, g40110.t1, g48658.t1, g48660.t1, g51446.t1, g64826.t1, g20314.t1, g598.t1, g42993.t1	24.416−53.802


(A)
Methylmalonyl-CoA
Fatty acid synthase
Acetyl-CoA
Methyl-branched fatty acids
Malonyl-CoA
Fatty acids
Fatty acid synthase
Desaturase
Unsaturated fatty acids
Elongase
Reductase
Cytochrome P450 decarbonylase
Methyl-branched alkane
n-alkane
monoene
Precursors
Fatty acyl-CoA
Aldehyde
Hydrocarbon
(B)
(C)
(D)
(E)
Nr: Non-rubbing
R3d: Rubbing behavior 3d
I9d: Isolation 9d
mRNA expression level of Desat (TPM)
MaxLFQ value of Desat
mRNA expression level of Cyp4g1 (TPM)
MaxLFQ value of Cyp4g1
n.s.
Nr
R3d
I9d
Host: C. japonicus (n=3)
Host: C. obscuripes (n=3)
Non-rubbing (n=5)
Rubbing 3d (n=5)
(F)
Color Key
DEGs of Non-rubbing vs Rubbing 3d (Top10)
DEGs of Rubbing 3d vs Isolation 9d (Top10)
The candidate CHC synthesis-related genes (non-DEGs)
Non-rubbing
Rubbing 3d (C. japonicus)
Rubbing 3d (C. obscuripes)
Isolation 9d (C. japonicus)
Isolation 9d (C. obscuripes)
No hit_g9808.t1
No hit_g34129.t1
No hit_g69521.t1
Ubiquitin-conjugating enzyme E2 G2_g61061.t1
No hit_g65054.t1
Haemolymph lipopolysaccharide-binding protein-like_g56481.t1
Defensin_g62621.t1
No hit_g994.t1
Uncharacterized protein_g63711.t1
Desaturase_g34819.t1
Lysosomal alpha-mannosidase isoform X1_g2502.t1
Hypothetical protein_g35963.t1
Uncharacterized protein_g63715.t1
Ribose-phosphate pyrophosphokinase 1 isoform X1_g7723.t1
Translation initiation factor if-2_g57346.t1
Trifunctional purine biosynthetic protein adenosine-3_g19442.t1
Histone H2B-like_g50866.t1
Arylphorin subunit alpha-like_g50909.t1
Hymenoptaecin_g63709.t1
Solute carrier organic anion transporter family member 2A1_g53887.t1
Fatty acid synthase_g30275.t1
Elongase_g39123.t1
Fatty acyl-CoA reductase_g58736.t1
Cytochrome P450 decarbonylase_g28547.t1

(cont'd)

Figure 4-11. The expression levels of CHC synthesis-related genes in the whole abdomen. (A) The synthesis pathway of CHC (modified from Chung & Carroll, 2015). (B&C) The expression levels of desaturase (*Desat*): g34819.t1 in mRNA from whole abdomen by transcriptome (B) and protein from fat body by quantitative analysis of key proteins using LC-MS analysis (C). (D&E) The expression levels of cytochrome P450 decarbonylase (*Cyp4g1*): g28547.t1 in mRNA from whole abdomen by transcriptome (D) and protein from fat body by quantitative analysis of key proteins using LC-MS analysis (E). In mRNA, *; Significant difference: FDR < 0.05 (the actual value: 0.000–0.008), n.s.; non-significant difference (the actual value: 1.000), n=3. In protein, one-way ANOVA, n.s.; non-significant difference (the actual value: 0.473–0.758), n=5. (F) The heatmap of expression levels of the top ten DEGs and the top-hit candidate CHC synthesis-related genes that were commonly observed regardless of the host species. This analysis was performed using the TPM that was converted to a Z score. The name of genes referred to in Figures 4-11B–E are enclosed in squares. Three days and nine days are respectively refered to as “3d” and “9d”.

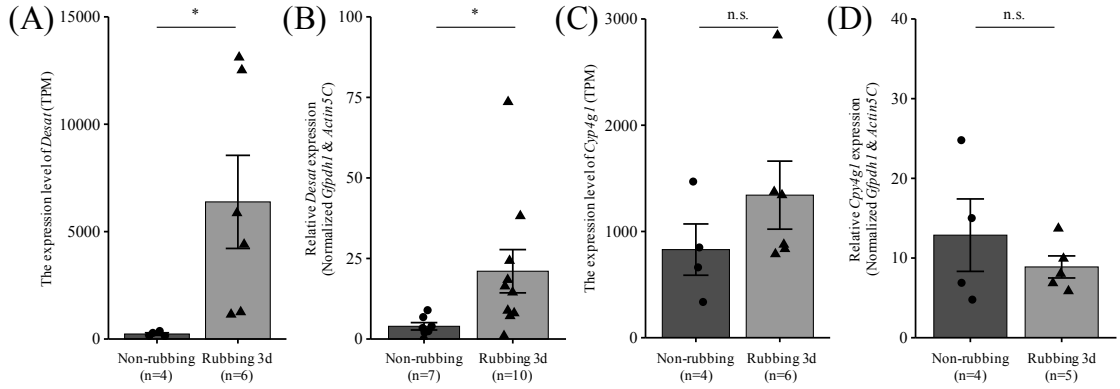


Figure 4-12. The levels of expression of CHC synthesis-related genes in fat bodies. (A&B) The levels of desaturase (*Desat*): g34819.t1 expression in fat bodies as analyzed by transcriptome [A, *; significant difference: FDR < 0.05 (the actual value: 0.000), n=4–6] and RT-qPCR [B, *; significant difference: p-value < 0.05 (the actual value: 0.031), Welch's t test, n=7–10]. (C&D) The levels of cytochrome P450 decarbonylase (*Cyp4g1*): g28547.t1 expression in fat bodies as analyzed by transcriptome [C, n.s.; non-significant difference (the actual value: 0.792), n=4–6] and RT-qPCR [D, n.s.; non-significant difference (the actual value: 0.454), Welch's t test, n=4–5]. All error bars indicate the standard error. Three days is refered to as "3d".

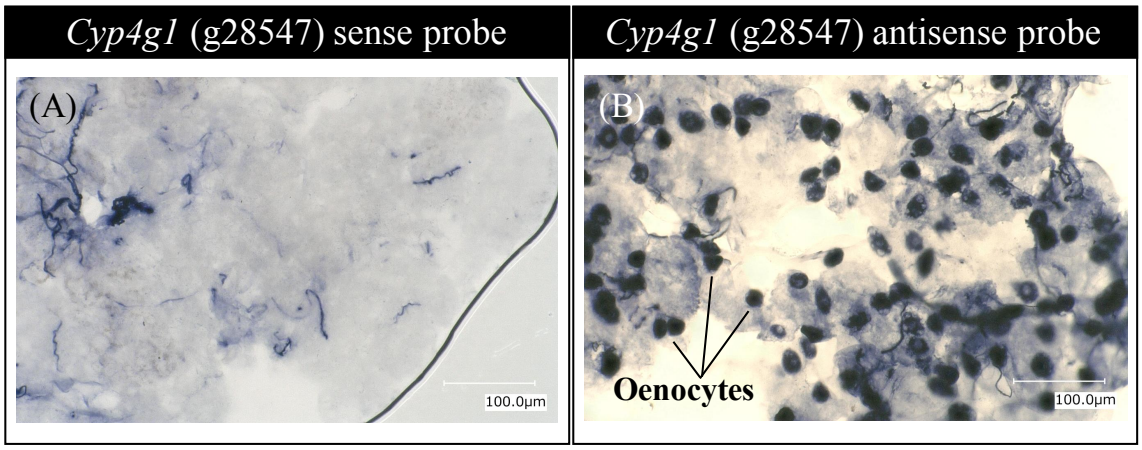


Figure 4-13. *in situ* hybridization of *Cyp4g1*. The sense (A) and antisense probe (B) were used in the fat body. The mRNA of *Cyp4g1* expressed on the oenocytes.

Table 4-8. The annotated DEGs.

Gene ID	Gene name	Top hit accession ID	The logFC of DEGs			
			Non-rubbing vs. Rubbing 3d		Rubbing 3d vs. Isolation 9d	
g587.t1	No hit	No hit	1.38	1.34	-	-
g994.t1	No hit	No hit	-5.88	-5.87	-	-
g2502.t1	Lysosomal alpha-mannosidase isoform X1	XP_025265136.1	-	-	2.11	1.43
g3009.t1	Fatty acid-binding protein homologue 7-like	XP_029664361.1	-	-	-1.21	-1.88
g3391.t1	Uncharacterized protein	XP_029666367.1	-	-	-1.68	-1.13
g4461.t1	Facilitated trehalose transporter Tret1-2 homologue	XP_011263555.1	-	-	-1.92	-3.06
g5567.t1	Uncharacterized protein	XP_011262925.2	-	-	-5.69	6.34
g6106.t1	Ubiquitin-conjugating enzyme E2 G2	XP_011636099.1	2.59	2.22	-	-
g7723.t1	Ribose-phosphate pyrophosphokinase 1 isoform X1	XP_029172996.1	-	-	2.37	1.96
g7979.t1	Hypothetical protein	EFN70423.1	-	-	-1.72	-1.21
g8968.t1	Sodium- and chloride-dependent betaine transporter	XP_011260796.1	-	-	-1.59	-2.23
g9111.t1	Fatty acid synthase-like	XP_011264667.2	1.84	1.63	-	-
g9808.t1	No hit	No hit	3.83	-7.67	-	-
g10769.t1	No hit	No hit	-	-	0.71	0.94
g11011.t1	Uncharacterized protein	XP_029665965.1	1.52	1.32	-	-
g11948.t1	Spidroin-2-like isoform X1	XP_029661708.1	-	-	-1.65	-1.95
g13735.t1	Hemocyte protein-glutamine gamma-glutamyltransferase	XP_011266432.1	-	-	-0.87	-1.26
g15692.t1	Metabotropic glutamate receptor isoform X1	XP_025266613.1	-	-	-2.41	-2.82
g16463.t1	Leucine-rich repeat-containing protein 15	XP_011253803.1	-	-	-0.88	-1.40

(cont'd)

g16588.t1	Fibrillin-1	XP_025262768.1	-	-	-1.18	-1.58
g19442.t1	Trifunctional purine biosynthetic protein adenosine-3	XP_025268113.1	-	-	1.50	1.91
g20432.t1	PREDICTED: uncharacterized protein	XP_012225306.1	-	-	-1.46	-0.93
g22437.t1	Arylsulfatase B isoform X1	XP_025270578.1	-	-	-0.92	-1.45
g23508.t1	Prostatic acid phosphatase isoform X2	XP_011255200.1	-	-	-1.33	-1.20
g24953.t1	No hit	No hit	-	-	-8.32	8.31
g25803.t1	No hit	No hit	-	-	2.67	3.49
g26264.t1	Multidrug resistance protein homologue 49 isoform X1	XP_011265189.1	-	-	-1.07	-1.09
g26732.t1	Solute carrier family 2, facilitated glucose transporter member 1 isoform X2	XP_011261955.2	-	-	1.16	1.18
g30275.t1	Fatty acid synthase-like	XP_025270749.1	-	-	-2.07	-1.23
g31112.t1	Scavenger receptor class B member 1	XP_011253015.1	-	-	-1.67	-2.06
g33203.t1	Uncharacterized protein	XP_011254770.1	-	-	-1.72	-1.47
g34129.t1	No hit	No hit	-2.56	-7.27	-	-
g34819.t1	Acyl-CoA Delta (11) desaturase	XP_025264947.1	2.63	1.73	-2.85	-1.93
g35207.t1	WEB family protein At4g27595, chloroplastic	XP_025268350.1	2.80	1.62	-	-
g35887.t1	Solute carrier organic anion transporter family member 2A1	XP_011255071.1	-	-	-1.38	-1.21
g35963.t1	Hypothetical protein	EFN74322.1	-	-	2.66	1.58
g35964.t1	Hypothetical protein	EFN74322.1	-	-	2.30	1.78
g38487.t1	Intraflagellar transport protein 43-like protein	KMQ94321.1	-	-	1.14	1.41
g38783.t1	MFS-type transporter SLC18B1	XP_011265521.1	0.53	0.61	-0.87	-1.51
g39515.t1	Calexcitin-2	XP_011256346.1	-	-	-1.44	-2.65

(cont'd)

g39681.t1	Sodium-coupled monocarboxylate transporter 1-like	XP_025261690.1	-	-	-1.12	-1.68
g40149.t1	NPC intracellular cholesterol transporter 2-like	XP_011258918.1	-	-	-3.96	-1.94
g40405.t1	Uncharacterized protein	XP_029668922.1	-	-	-1.09	-1.38
g46969.t1	Uncharacterized protein	XP_011264753.1	-	-	-0.83	-1.19
g47732.t1	Lipid storage droplets surface-binding protein 1	EFN73399.1	-	-	-1.00	-1.05
g49055.t1	Glutamic acid-rich protein-like	XP_011259581.1	-	-	1.20	0.96
g49288.t1	Pheromone-binding protein Gp-9-like isoform X2	XP_029660118.1	-	-	-3.11	-3.34
g50866.t1	Histone H2B-like	XP_011632649.1	-	-	-1.71	-1.74
g50909.t1	Arylphorin subunit alpha-like	XP_029170871.1	-	-	3.02	-3.88
g51070.t1	No hit	No hit	-	-	-2.40	-8.86
g51590.t1	No hit	No hit	-	-	2.18	4.47
g51996.t1	Venom acid phosphatase Acph-1-like	XP_025271241.1	-	-	-1.27	-1.59
g52645.t1	Probable ascorbate-specific transmembrane electron transporter 1	XP_025266889.1	-	-	-1.81	-3.55
g52952.t1	Mitochondrial pyruvate carrier 1-like	XP_029665361.1	-	-	-1.37	-1.17
g53082.t1	Sodium-coupled monocarboxylate transporter 1 isoform X1	XP_025271097.1	-	-	-1.29	-1.13
g54876.t1	L-lactate dehydrogenase	XP_011265340.1	-	-	-1.10	-1.29
g56481.t1	Haemolymph lipopolysaccharide-binding protein-like	XP_011253802.2	2.16	2.23	-1.66	-2.43
*g57012.t1	Cytochrome P450 4g15	XP_011267879.1	1.48	1.54	-1.28	-1.85
g57346.t1	Translation initiation factor if-2	KMQ96767.1	-	-	-1.42	-1.66
g57976.t1	No hit	No hit	-	-	-5.96	-6.55
g58186.t1	Feline leukaemia virus subgroup C receptor-related protein 2-like	XP_025261827.1	-	-	-1.02	-1.05

(cont'd)

g58387.t1	Inositol monophosphatase 1	XP_011257136.1	-	-	-1.54	-1.36
g58775.t1	Maltase 1-like	XP_025263040.1	-	-	-2.39	-4.58
g59001.t1	Cytosolic 10-formyltetrahydrofolate dehydrogenase	XP_011251043.2	-	-	-2.11	-2.49
g59329.t1	Uncharacterized protein	XP_025266319.1	-	-	1.29	-3.44
g60423.t1	No hit	No hit	-	-	-6.32	-4.87
g60655.t1	No hit	No hit	-	-	-2.50	-3.94
g61485.t1	Synaptic vesicle glycoprotein 2B	XP_011254928.1	-	-	-0.98	-1.21
g61864.t1	Phospholipase B1, membrane-associated	XP_011257663.3	2.51	2.02	-	-
g62475.t1	No hit	No hit	-	-	10.03	6.56
g62621.t1	Defensin	XP_025262447.1	7.13	7.38	-6.52	-8.17
g63022.t1	No hit	No hit	-	-	1.31	1.05
g63075.t1	No hit	No hit	-	-	1.03	1.03
g63707.t1	Uncharacterized protein	XP_025268004.1	-	-	-6.57	-8.46
g63709.t1	Hymenoptaecin	KMQ95866.1	6.69	7.80	-7.72	-8.92
g63711.t1	Uncharacterized protein	XP_025268004.1	6.57	7.74	-7.44	-8.97
g63714.t1	Uncharacterized protein	XP_025268004.1	6.32	7.69	-7.34	-8.98
g63715.t1	Uncharacterized protein	XP_025268004.1	6.52	7.96	-7.61	-9.11
g64630.t1	Cuticle protein 2-like	XP_025269546.1	-	-	-1.60	-1.86
g64805.t1	Abnormal long morphology protein 1-like	XP_029162544.1	-	-	-2.57	3.39
g65054.t1	No hit	No hit	-6.06	-6.04	-	-
g65360.t1	No hit	No hit	-	-	-1.81	-2.89
g67717.t1	Facilitated trehalose transporter Tret1	XP_025268384.1	-	-	-0.98	-1.28
g67726.t1	Mediator of RNA polymerase II transcription subunit 12	XP_025264718.1	-1.80	4.11	-	-
g69521.t1	No hit	No hit	4.63	3.50	-	-
g69762.t1	Larval cuticle protein A2B-like	XP_011260338.1	-	-	-1.39	-1.74

(cont'd)

The predicted gene names of the DEGs were commonly observed regardless of the host species (FDR < 5%). The logFC is written for the comparison group in which changes in expression were observed. *One candidate for cytochrome P450 decarbonylase (*Cyp4g1*), g57012.t1, was detected by RNA-Seq in the whole abdomen. However, the candidate was omitted from the discussion in the text because its pattern of expression was different in the fat body. Three days and nine days are respectively refered to as “3d” and “9d”.

4-4 Discussion

Rubbing behavior was shown to disguise the host-like CHC profile and prevent the aggression of hosts, and this was demonstrated through replicating rubbing behavior in the laboratory, testing the nestmate-recognition behavior by hosts, and performing GC-MS analysis with the newly mated *P. lamellidens* queen against its host worker. In addition, the newly mated *P. lamellidens* queens acquired and maintained artificially-applied labeling substances from the host workers by performing rubbing behavior, while gene expression profiling showed no change in the expression of cytochrome P450 decarbonylase (*Cyp4g1*), which is a CHC synthesis-related factor, during rubbing behavior. These results suggest that the newly mated *P. lamellidens* queens directly acquire the hosts' CHC through rubbing behavior and deceive the technique of nestmate recognition by hosts through employing chemical disguises.

In a previous rearing study, a reduction in aggressive behavior was observed in host workers towards the newly mated *P. lamellidens* queen after rubbing behavior was performed (Kohriba, 1963). My results demonstrate that the effect is due to chemical disguise (Figure 4-2) and quantitatively indicate that versatility promotes this disguise in both host species (*C. japonicus* and *C. obscuripes*), which exhibit different CHC profiles (Figures 4-2, 4-3, 4-4, and 4-5, Tables 4-2 and 4-3). In addition, I revealed that the newly mated *P. lamellidens* queens possessed only a minute amount of CHCs before the rubbing behavior (Figure 4-2D, Tables 4-2 and 4-3), and this quantity was significantly increased after the rubbing behavior for *C. japonicus* (Figure 4-2D). Obtaining a new CHC is natural and simple when the baseline is limited, and this observation also supports the idea of chemical camouflage (acquirement of CHC). Since a low base quantity of CHC has been observed in other social parasitic ants, I expect it to be one of their general strategies (d'Ettorre & Errard, 1998; Johnson et al., 2001; Martin et al., 2007; Tsuneoka & Akino, 2012). The tracing assay with the labeled substances revealed that the newly mated *P. lamellidens* queen acquires labeled substances from its host workers by performing rubbing behaviors (Figures 4-6B, 4-7, and 4-8B–D). This result also confirmed that the newly mated *P. lamellidens* queens directly obtained host CHCs through rubbing behaviors. Previous studies reported that ants exchange CHC through performing trophallaxis and grooming with nestmates, and through these behaviors, the CHC profile becomes standardized within the colony (Dahbi et al., 1999; Soroker et al., 1994 & 1995). The newly mated *P. lamellidens* queen also grooms the host while

performing rubbing behaviors, and the queen probably acquires the CHC of hosts during this process (Kubota, 1974).

Isolation rearing revealed that the newly mated queen, which was isolated from its hosts for up to nine days, could maintain the disguised CHC profile (Figures 4-8A–C, 4-8E, and 4-10). This result suggests that the newly mated queen could maintain the amount and profile of the CHC that was acquired from the host. It is known that ants store CHC in the PPGs in their heads (Soroker et al., 1994 & 1995). I also confirmed that the PPGs of newly mated *P. lamellidens* queens contain host CHC and labeled substances from its labeled host workers after performing rubbing behaviors (Figures 4-8D and 4-9). The hydrocarbon profile found in the PPGs was similar to that of the host CHC, and the labeled substances applied to the host were also detected in the PPGs, suggesting that the newly mated *P. lamellidens* queen also supplies the acquired host CHC to its PPGs. Most likely, the newly mated queen enables the disguised CHC profile to be maintained by storing the acquired CHC in the PPGs.

Expression analysis was performed to verify the chemical mimicry, and except for the mRNA of desaturase (*Desat*) (but not the protein level), no changes were observed in the expression of genes in the pathways related to CHC synthesis before and after rubbing behavior (Figures 4-10 and 4-11, Tables 4-6, 4-7, and 4-8). This result suggests that the biosynthesis of CHC, i.e., chemical mimicry, does not occur in the newly mated *P. lamellidens* queens. Among the CHC synthesis-related genes, the expression of cytochrome P450 decarbonylase (*Cyp4g1*) (Koto et al., 2019), which is responsible for the final modification of CHC precursors and has an expressional level that correlates with the amount of CHC synthesized, was not detected at the mRNA or protein level (Figures 4-11D–E and 4-12CD, Tables 4-6, 4-7, and 4-8). Since the expression of cytochrome P450 decarbonylase (*Cyp4g1*) did not change despite the apparent increase in the amount of CHC after rubbing behavior, I expected that no new biosynthesis of CHC would occur, at least during the rubbing behavior. The expression level of Desaturase (*Desat*), which is involved in the construction of CHC variants (Chung & Carroll, 2015), changed at the mRNA level but not at the protein level (Figures 4-11BC and 4-12AB, Tables 4-6, 4-7, and 4-8). Based on the above observations, I expected that the newly mated *P. lamellidens* queen would achieve chemical disguise by performing chemical camouflage (acquiring CHC) rather than chemical mimicry (CHC biosynthesis) through rubbing behavior.

In general, socially parasitic ants and their host are considered to be phylogenetically closely related, which is known as Emery's rule (Emery, 1909; Hölldobler and Wilson, 1990). The reason why the application of Emery's rule to some socially parasitic ants is thought to be because it is easy for the parasitic ants to carry out the chemical disguise (Blatrix & Sermage, 2005; de la Mora et al., 2020; Huang & Dornhaus, 2008). However, some parasitic species deviate from this rule, such as *P. lama* and *P. loweryi*, which host distantly related *Diacamma* sp. and *Rhytidoponera* sp. (Formicidae: Ponerinae) at the subfamily level (Maschwitz et al., 2000, 2003, & 2004; Witte et al., 2009). *P. lama* is estimated as avoiding the nestmate discrimination of its host by other means (e.g., to use the appeasement substance) without CHC disguise, perhaps because it is phylogenetically quite distant from its host (Witte et al., 2009). *P. lamellidens* also allows the phylogenetically distant species (*C. japonicus* etc.) as its host (although they belong to the same clade at the subfamily level), and such socially parasitic ants are expected to have difficulty avoiding the nestmate discrimination of their host without experiencing some special event. The acquisition of host CHC by rubbing behavior, as performed by the newly mated *P. lamellidens* queen, may be an example of this. The shampoo ant (*Formicoxenus* sp.), which allows its host species of a different genus but the same at the subfamily level (Ojeda et al., 2021), is also presumed to acquire the host CHC through the unique behavior similar to the rubbing behavior of *P. lamellidens* (Lenoir et al., 1997). As in the above examples of *Polyrhachis* species, how the nestmate discrimination of the host ants is deceived may vary according to the phylogenetic distance between parasite and host. This question could be answered by examining the chemical disguise and its mechanism (chemical camouflage and/or chemical mimicry) through research approaches such as mine (e.g., CHC analysis, tracing assay of the labeled substances, and the gene expression analysis) against several parasitic ants which follow or do not follow the Emery's rule.

I used a combination of experimental techniques to reveal the chemical disguise strategy of the newly mated *P. lamellidens* queen in the early stages of social parasitism. In addition, I believe that this study succeeded in elucidating some of the previously obscure mechanisms that are involved in the chemical disguise of socially parasitic ants.

Chapter 5

Final remarks

5-1. Biology of social parasitism (*P. lamellidens*)

This dissertation aimed to elucidate the chemical disguise mechanism of socially parasitic ants of *P. lamellidens*. First, the interactions between *P. lamellidens* and their associated organisms (myrmecophiles and hosts) are illustrated in Chapters 2 and 3. Chapter 2 investigates the symbiotic relationship of *M. katsurai* and *P. lamellidens* using field research and a rearing experiment (Iwai et al., 2016). Field research shows *M. katsurai* larvae in *P. lamellidens* nests, suggesting that *M. katsurai* is myrmecophile with *P. lamellidens* as a host. Furthermore, morphological observations using the puparium revealed that the conventional classification system of *M. katsurai* based on adult morphology was valid. Chapter 3 presents detailed field observations, behavioral tests, and rearing experiments, showing that *C. obscuripes* is a host of temporary social parasites (Iwai et al., 2021). Colonies with *P. lamellidens* queens, workers, and larvae were intermixed with *C. obscuripes* workers in the field. Behavioral tests showed that workers of both species in mixed colonies did not attack each other and maintained nestmate recognition ability, suggesting a collaborative nestmate relationship. Furthermore, a rearing experiment confirmed social parasitism by *P. lamellidens* among *C. obscuripes* by producing a mixed brood-producing colony. These are the first field and laboratory records of temporary social parasitism involving *P. lamellidens* and *C. obscuripes*. Finally, Chapter 4 investigates the chemical disguise mechanisms of *P. lamellidens* (Iwai et al., 2022) based on the findings of Chapters 2 and 3. A comparative analysis of CHC in *P. lamellidens* and its host revealed that the CHC profile in *P. lamellidens* changes to a similar profile to that of the host through rubbing behavior. In addition, *P. lamellidens* acquired artificially applied labeling substances from host workers through their rubbing behavior, whereas gene expression profiling showed that the expression of CHC synthesis-related genes did not change during this behavior. These results suggest that the role of rubbing behavior is to disguise the CHC profile to those similar to the host by directly acquiring the host CHC. Given the abovementioned studies, it was possible to clarify the existence of organisms having a symbiotic relationship with *P. lamellidens*, identify the host range, and determine the chemical disguise mechanisms performed by this species (Figure 5-1).

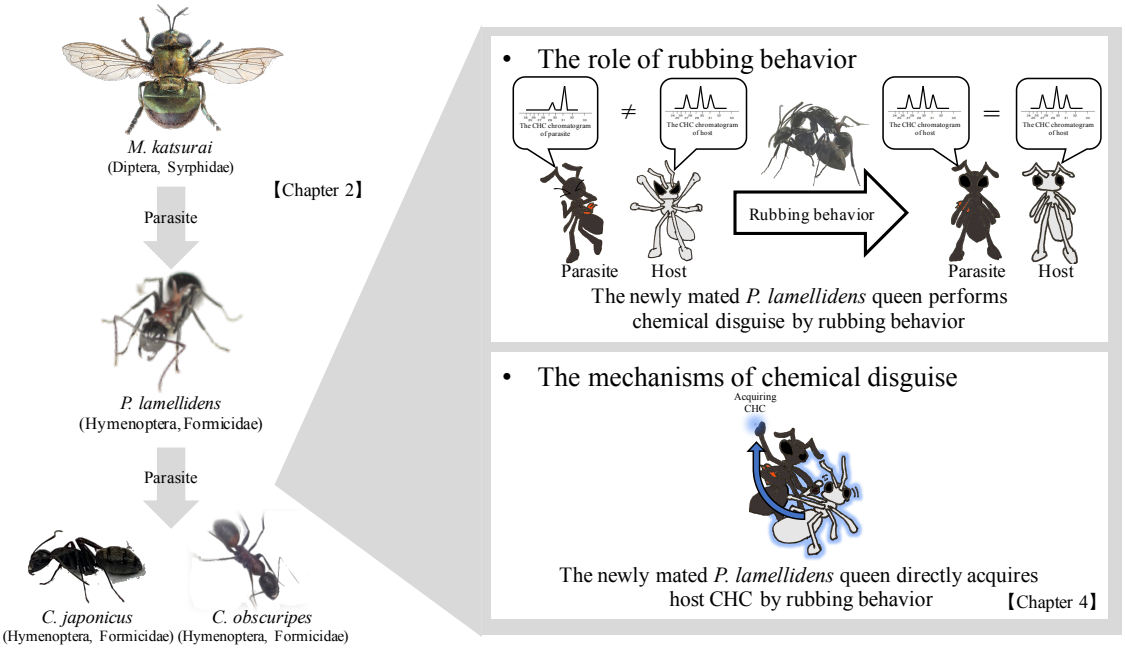


Figure 5-1. The summary of this dissertation. *M. katsurai* utilizes *P. lamellidens* as its host (Chapter 2), and *C. obscuripes* is an alternative host of *P. lamellidens* (Chapter 3). The role of rubbing behavior by the newly mated *P. lamellidens* queen is the chemical disguise, and its mechanism is directly acquiring host CHC (Chapter 4).

5-2. Evolutionary background of social parasitism and chemical disguise

This dissertation discusses the hypotheses below regarding the factors leading to multiple occurrences of socially parasitic ants in various ant taxa. The chemical disguise mechanism of *P. lamellidens* is a simple disguise strategy that can be achieved simply by acquiring CHCs of the host without much intrinsic CHCs. It is a relatively low-cost and easy-to-acquire strategy for deceiving the vulnerable nestmate discrimination of ants using the CHC profile. A low base quantity of CHCs has been observed before chemical disguise in other socially parasitic ants (e.g., the *Polyergus* genus), which is one of their general strategies (d'Ettorre & Errard, 1998; Johnson et al., 2001; Martin et al., 2007; Tsuneoka & Akino, 2012). Furthermore, some socially parasitic ants have been observed to avoid the host nestmate discrimination by permanently losing most original CHCs (chemical insignificance), which is a so-called stealth state (de la Mora et al., 2020). This suppression of CHC biosynthesis is a frequent feature of socially parasitic ants. The ability to deceive the nestmate discrimination of other ant species by acquiring such a simple strategy may be one factor behind the multiple occurrences of socially parasitic ants in various ant taxa.

One possible factor for the origin of social parasitism is establishing a polygynous state of a newly mated queen (Buschinger, 2009). Polygyny is the existence of multiple queens in one colony. The relatedness coefficient among queens and the timing of polygynous states vary (e.g., permanently maintaining a polygynous state, staying in a polygynous state only at the early stage of colony foundation, and newly mated queens returning to their original nests) (Hölldobler & Wilson, 1990). Among these, the return of the newly mated queens to their original nests is presumed to be the origin of the invasion of other ant colonies by the socially parasitic ants. Such a polygynous condition has been observed in the socially parasitic ants (*P. lamellidens*) when the host has been killed to complete parasitism (Materials and Methods of Chapters 2 and 4). In addition, at the point of host colony takeover in the early stages of social parasitism, multiple newly mated *P. lamellidens* queens, regardless of kinship, cooperatively kill the host queen (Figures 5-2 and 5-3). Furthermore, the newly mated queens have been identified in the procession of *P. lamellidens* colonies. The abovementioned information suggests that *P. lamellidens* is a polygynous ant and that the newly mated queens return to their nest. This knowledge could indicate nest return as the origin of social parasitism, as clarified in previous studies (Buschinger, 2009). The CHC profile unification of each queen through

chemical disguise at the time of the invasion of the host colony is a factor contributing to the establishment of polygyny. For example, when invading a host colony, the newly mated *P. lamellidens* queens perform the chemical disguise on the host workers (Chapter 4). However, if multiple newly mated queens simultaneously invade a particular host colony, the CHC profiles of each queen will all be identical. The newly mated *P. lamellidens* queens are expected to recognize each other as the host workers because they have few original CHCs and disguise the host-like CHC profile. Accordingly, they can kill the host queen and oviposition cooperatively, as shown in Figures 5-2 and 5-3.

5-3. Combination approach of chemical ecology and molecular biology

This dissertation elucidates the basic ecological information of *P. lamellidens* and some chemical disguise mechanisms in this species using field research, measuring the CHC profile of ants, performing a tracing assay with labeled substances, and analyzing gene expression levels. Most previous studies aiming at elucidating the chemical disguise mechanisms have verified the persistence of disguised CHCs by isolating myrmecophile from its host (Akino et al., 1996; Scarparo et al., 2019; Vander Meer & Wojcik, 1982; von Beeren et al., 2011 & 2012). These studies revealed whether the CHC profile returns to its predisguised state when myrmecophiles are isolated from their hosts. Researchers believe that chemical disguise is performed by chemical camouflage (acquirement of CHC) in the former case and chemical mimicry (CHC biosynthesis) in the latter (Akino et al., 1996; Scarparo et al., 2019; Vander Meer & Wojcik, 1982; von Beeren et al., 2011 & 2012). However, the results of isolation rearing and tracing assay of the labeled substances in this study showed that the newly mated *P. lamellidens* queen maintained CHC disguise via chemical camouflage for a certain period even in the absence of the host. These results suggest that even if the parasite maintains a disguised CHC profile for a long time through isolated rearing, it is not caused by CHC biosynthesis (chemical mimicry). An appropriate experimental system corresponding to the parasite should be constructed (e.g., tracing assay with labeled substances and analyzing gene expression levels) to elucidate chemical disguise mechanisms and perform isolation rearing.

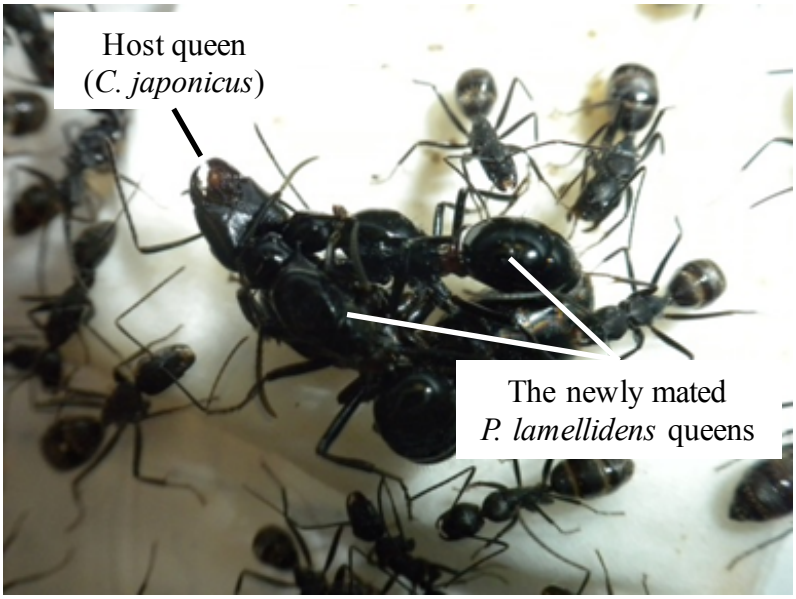


Figure 5-2. The killing of a host queen by newly mated *P. lamellidens* queens. Newly mated *P. lamellidens* queens bite the neck of the *C. japonicus* queen in a laboratory condition.

Figure 5-3. Eggs laid by the parasite queens (*P. lamellidens*) in the host (*C. japonicus*) colony. *P. lamellidens* eggs were laid after the killing of the *C. japonicus* queen.

5-4. Deception of the nestmate discrimination after colony invasion

Although this dissertation focuses on how the newly mated *P. lamellidens* queen disguises its CHCs through the rubbing behavior, questions remain concerning the CHC maintenance mechanism acquired from the host. Furthermore, how the queen and workers maintain the deception of the nestmate discrimination by the host workers even after the host queen is successfully killed remains unclear.

It is known that myrmecophiles disguise their CHC by chemical camouflage and lose their CHC within approximately nine days (von Beeren et al., 2011 & 2012), making the case of *P. lamellidens* unique. PPGs is expected to be one factor in the maintenance of the acquired CHC. As revealed in this dissertation, the newly mated *P. lamellidens* queen stores CHC acquired from the host in PPG. The newly mated *P. lamellidens* queen is estimated to maintain her disguised CHC profile even in the absence of a host by continuously replenishing acquired CHCs, which are gradually lost from the cuticle of PPG. The hypothesis could be verified by isolating the postdisguised newly mated *P. lamellidens* from the host for some time and examining the transitions in the amount of CHCs present on cuticles and the PPG at each time point during the isolation period.

The rubbing behavior of the newly mated *P. lamellidens* queen is performed only at the initial parasitism stage. It is no longer performed when she takes over the host colony and starts oviposition (Japanese Ant Database Group, 2003; Kohriba, 1963; Sakai, 2000; Yano, 1911). Therefore, the newly mated *P. lamellidens* queen would either maintain the CHCs acquired during the rubbing behavior or switch to other methods to continue chemical disguise. Ants exchange their CHCs through nutrient exchange (Dahbi et al., 1999; Soroker et al., 1994 & 1995). As the newly mated *P. lamellidens* queen continues to exchange nutrients with host workers after oviposition (Kohriba, 1963; Sakai, 2000), the queen is probably acquiring host CHCs through the nutrient exchange.

The newly produced *P. lamellidens* workers coexist temporarily (until the host workers die) with the host workers. Therefore, like newly mated queens, *P. lamellidens* workers also probably somehow deceive the nestmate discrimination system of the host ants. Previous studies have shown that workers of several socially parasitic ants, like their queen, deceive the nestmate discrimination of the host through various strategies, such as disguising the CHC profile, adapting the CHC profile of the host to their own, or not having much original CHCs (Fwłodarczyk & Szczepaniak, 2017; Lambardi et al., 2007;

Liu et al., 2003; Martin et al., 2007). *P. lamellidens* workers may deceive their host ants using one of the abovementioned methods.

The newly mated *P. lamellidens* queen kills the host queen after the successful invasion of the host colony (Japanese Ant Database Group, 2003; Sakai, 1996). This behavior has also been observed in the newly mated *Polyergus* queens; they disguise the CHC profile of the host queen during the killing (d'Ettorre & Errard, 1998; Johnson et al., 2001; Tsuneoka & Akino, 2012). In addition, the newly mated *P. lamellidens* queen probably disguises the CHC profile of its host queen at this time, like *Polyergus*. In several ant species (including *C. japonicus*, *P. lamellidens* host), the CHC profile differs between castes, which may be involved in queen recognition and reproductive inhibition in the worker caste (Dietemann et al., 2003; Endler et al., 2004; Heinze et al., 2002; Hojo et al., 2009; Liebig et al., 2000; Sharma et al., 2015). Therefore, like in host queens, disguising the CHC profile is probably advantageous in keeping host workers under control.

5-5. Future prospects

This dissertation successfully revealed the mechanism of the chemical disguise in the newly mated *P. lamellidens* queen, which disguises by acquiring the CHC of its host without the biosynthesis of its own CHC as much as possible. Such a chemical disguise mechanism, seemed simpler and relatively an easier strategy than the biosynthesis of a new CHC similar to the host. Therefore, I speculated that the acquisition of chemical disguise is one of the factors responsible for the evolution of socially parasitic ants in various ant taxa. As mentioned earlier, the suppression of CHC production was frequently observed in socially parasitic ants, thus these parasitic ants likely employed the same disguise strategy as revealed in this dissertation. By conducting experiments such as those described in this dissertation (e.g., tracing assay with labeled substances, analyzing gene expression levels) on various socially parasitic ants across taxonomic groups and detailed parasitic strategies (e.g., dulosis, xenobiosis), it will be possible to verify the aforementioned hypotheses, which in turn will lead to a better understanding of the evolutionary background of social parasitism.

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