**Prevalence, geographic distribution, and transmission pathway of Camponotus yamaokai virus**

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**Abstract**

The relationship between hosts and viruses is influenced by various factors. One potential factor is sociality. In social organisms such as ants, the interaction between hosts and viruses might differ from those of solitary organisms due to their unique ecology. We previously isolated a double-stranded RNA toti-like virus, Camponotus yamaokai virus (CYV), from the arboreal ant *Camponotus yamaokai*. The ant exhibits a polygynous colony structure with multiple queens and within-nest mating behaviors. Such unique ecological traits may have driven the evolution of a distinctive relationship with the virus. However, the biological characteristics of CYV have not been sufficiently studied. In this study, we investigated the biological characteristics of CYV through rearing experiments and field surveys. As a result, no horizontal transmission was detected between workers and broods. There were no significant differences in prevalence between castes. CYV was detected at all seven surveyed locations, with location prevalence ranging from 60% to 95%. The high CYV prevalence across the host's distribution range indicates that the geographical distribution of CYV aligns with that of its host. These results suggest that CYV has spread throughout the host population, primarily relying on vertical transmission.

**Keywords:** Totiviridae, ant, social insect, prevalence, vertical transmission

## Introduction

The spatial distribution and the frequency of interactions between individuals can influence the prevalence of viruses [1]. Social organisms, which live at high densities and engage in direct contact or the exchange of materials through social interactions, may be more prone to virus transmission. They, however, have developed preventive mechanisms at both social and individual levels to control the spread of infections [2]. This suggests that social organisms, such as ants, may exhibit unique host-virus relationships that are not observed in solitary organisms. Previous studies on the interaction between ants and viruses have been conducted, with significant progress made in those focusing on invasive species such as fire ant (*Solenopsis invicta*) and Argentine ant (*Linepithema humile*) [3, 4]. Given the diverse behaviors and social structures across different ant species [6, 7], the relationship between viruses and their hosts might be linked to such ecological aspects of ants.

A toti-like virus, Camponotus yamaokai virus (CYV), was reported from the arboreal ant *Camponotus* (*Myrmamblys*) *yamaokai* [8]. *Totiviridae* are double-stranded RNA viruses with two open reading frames (ORFs) coding for capsid protein (CP) and RNA-dependent RNA polymerase (RdRp), respectively. They have been known to infect yeasts, fungi, and protozoa [9]. Recently, toti-like viruses have been isolated from a wide range of hosts, including plants, shrimps, mosquitoes, and fish [10–13]. Toti-like viruses have also been detected in invasive ants, such as the fire ant (*Solenopsis invicta*) and the Argentine ant (*Linepithema humile*) [14, 15]. *C. yamaokai*, the host of CYV, is an arboreal ant species distributed in Honshu, Shikoku, and Kyushu in Japan, nesting in cavities of small branches[16]. The worker ant of this species ranges from 3.5 to 4.5 mm in length and exhibits dimorphism: major and minor workers. This species shows a high degree of polydomy, and individuals freely move among the nests in the same colony [16]. They exhibit a polygynous colony structure with multiple queens, where alate females engage in within-nest mating with males. Virus particles have been observed in the ovaries and oocytes of CYV-infected queens, suggesting reproductive vertical transmission via eggs [8]. Such a unique ecology of the host may have driven the evolution of a distinctive relationship with the virus. However, the biological characteristics of CYV have not been sufficiently studied.

In this study, we investigated the biological characteristics of CYV, including its transmission routes, prevalence, and distribution. First, *C. yamaokai* was collected from seven sites, and two of these sites were used to investigate whether there was a caste difference in CYV infection. Then, the intranidal infection rates at all sites were examined. Next, workers of *C. nawai*, which were not infected with CYV [8], were used to rear *C. yamaokai* eggs to adulthood. This allowed us to test whether CYV, transmitted through reproductive vertical transmission via parental gametes, could be maintained to adulthood without further CYV infections. Finally, source colonies of *C. yamaokai* with different CYV infection statuses were created. Using these source colonies, experimental colonies with different CYV infection statuses in eggs and rearing workers were established. This experiment aimed to examine whether food exchange (trophallaxis) between larvae and workers could lead to

behavioral horizontal transmission of CYV.

**Materials and methods**

1. Ant collection

The host ant, *Camponotus yamaokai*, was collected from seven locations in Japan including Miyagi, Saitama, Tokyo, Kanagawa, Shizuoka, Miyazaki, and Kagoshima (Fig.1). We brought back the ants along with the branches containing their nests to the laboratory. The collected nests were kept in complete darkness at 25 ± 1°C and fed twice a week with artificial bee food (Bee Hatcher, FEED ONE Co.), house cricket (*Acheta domesticus*) and 6% honey solution.

2. Real-Time PCR

To detect the virus, the following procedures were performed. RNA was extracted from the entire body of the insects using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Shiga, Japan) according to the manufacturer's protocol with a modification: the reverse transcription reaction mixture contained 20% dimethyl sulfoxide for chemical denaturation. The presence of the virus was determined by real-time PCR using GoTaq® qPCR master mix (Promega, WI, USA) according to the manufacture's protocol, with the Thermal Cycler Dice® Real Time System II (Takara Bio). Primer sequences used in real-time PCR were as follows: for ORF1 of CYV (GenBank accession number LC026053), qPCR_F215-234 (5'- TCTGACCAGTCAAGCGACAG-3') and qPCR_R323-304 (5'-GTCTTGTCCGCAAAATGTTG-3'); for RdRp motif of CYV, Motif3-F (5'-ATAACAACAACAATGGCATC-3') and Motif4-R (5'-CAAACCGTTCACCGTCAATA-3'); for β-actin of the ant (GenBank accession number LC492075.1), Yam_βactinF (5'-ACTGGGACGACATGGAAAAG-3') and Yam_βactinR (5'-AGTCATCTTCTCGCGATTGG-3'). β-actin was used to verify nucleic acid integrity. The reaction conditions started with denaturation at 94°C for 2 minutes, followed by 45 cycles of 94 °C for 30 seconds, 60°C for 30 seconds, and 72 °C for 30 seconds. Fragment specificity was checked in a melting curve. The samples showing an amplification with either or both CYV specific primer pairs were determined to be positive for the virus.

3. CYV prevalence

The CYV infection status was examined individually for all castes (21 queens, 22 major workers, 27 minor workers, 8 alate females and 16 males) from 27 nests collected in Kanagawa, and for 15 queens, 15 major workers, and 101 minor workers from 7 nests collected in Tokyo. For the alate females and males collected in Tokyo, virus detection was not performed due to an insufficient number of specimens. Additionally, to examine the CYV infection status within the nests, 15 minor workers were

sampled from five nests across all seven locations.

4. Interspecific fostering experiment

To investigate whether CYV undergoes reproductive vertical transmission, we conducted a interspecific fostering experiment in which *C. yamaokai* eggs were reared by workers of *C. nawai*. Since *C. nawai* is not infected with CYV [8], this experimental setup minimizes possibility of infection other than reproductive vertical transmission. Minor workers of *C. nawai* collected in Shizuoka, Japan, were used to create three experimental colonies. Each experimental colony consisted of three major workers and 12 minor workers as nurse individuals. All these workers were marked with paint on their mesonotum. Approximately 100 eggs from five nests of *C. yamaokai* collected in Yokohama were introduced into each experimental colony. The infection rate in these eggs should be similar to that observed in the Kanagawa site. These experimental colonies were reared until the introduced eggs emerged into adult *C. yamaokai*. Rearing conditions of the experimental colonies containing *C. nawai* workers and *C. yamaokai* eggs were the same as those described above for the maintenance of *C. yamaokai*. Adult *C. yamaokai* that emerged from the introduced eggs were distinguished from *C. nawai* by the absence of markings. The newly emerged *C. yamaokai* were stored at -80°C until verification of the CYV infection. To determine whether *C. nawai* adults became infected with CYV through the rearing process, the infection status of the *C. nawai* nurse individuals was also examined. From each experimental colony where eggs emerged, three *C. nawai* workers were randomly collected at the end of the experiment and examined for CYV infection.

5. Intraspecific fostering experiment

5.1 Creation of source colony

This experiment investigated whether behavioral horizontal transmission occurs between nurse workers and broods of *C. yamaokai*. For this purpose, it was necessary to establish source colonies with different CYV infection statuses. Six experimental colonies were established from nests of *C. yamaokai* collected in Kanagawa, each consisting of one queen and five workers. The workers were marked with paint on their mesonotum. Afterward, the colonies were reared until three offspring workers of the queen emerged, at which point the initially marked workers were removed. The colonies were then reared for two years until the number of offspring workers increased to about ten. All emerging workers were collected and examined for CYV infection.

5.2 Fostering experiment

To investigate whether CYV is horizontally transmitted from infected to uninfected hosts through trophallaxis among nestmates, four source colonies were created using the methods described in 5.1. The colonies were reared until the number of workers reached about 15. Three workers from each

source colony were sampled to check for the presence of the CYV, confirming that the virus infection status among workers was consistent within each colony. From each source colony, 10 minor workers were taken to create experimental colonies. If the experimental colony was CYV-positive (infection rate ~ 100%), eggs were taken from a CYV-negative source colony (infection rate = 0%), and vice versa (Fig.2). This ensured that in the experimental colonies, workers cared for broods with a different virus infection status than themselves. The experimental colonies were then kept for three months, until the introduced eggs developed into adults. All adult ants that emerged, as well as the nurse workers, were collected and examined for the presence of the virus.

**Results**

1. Caste differences in CYV prevalence

In the ants collected in Kanagawa, the CYV prevalence of all castes was similar, around 80% (Fisher's exact test, $p = 0.897$; Fig. 3; Supplemental table S1). Additionally, in the Tokyo population, the CYV prevalence of queen and worker castes (major and minor workers) were similar, around 80% ($p = 0.927$).

2. CYV prevalence

The CYV prevalence was investigated at seven locations across Japan. Most of all nests had prevalence exceeding 60% (Fig.4). In one nest collected from Tokyo, the prevalence was as low as 6.7%. In one nest from Kagoshima, the CYV was not detected in any of the individuals analyzed. The average CYV prevalence within locations was over 60%. The average prevalence per colony (mean ± SE) for locations was as follows: Miyagi: 97.3 ± 2.4%, Saitama: 78.8 ± 2.2%, Tokyo: 78.7 ± 16.2%, Kanagawa: 77.3 ± 6.8%, Shizuoka: 94.7 ± 2.2%, Miyazaki: 92.0 ± 3.5%, Kagoshima: 61.3 ± 14.9%.

3. Interspecific fostering experiment

In three experimental colonies with *C. nawai* workers, a total of 267 *C. yamaokai* eggs were introduced (Table 1). Most of the introduced eggs disappeared from the experimental colonies. No eggs introduced into colony 1 developed into adults, but a total of 12 minor workers emerged from colonies 2 and 3. When these workers were examined for CYV infection, 9 were positive and 3 were negative, with prevalence of 75%, which was similar to the prevalence of the ants in Kanagawa from which the eggs were sourced. CYV was not detected in any of the *C. nawai* workers collected from colonies 2 and 3.

4. Intraspecific fostering experiment

4.1 Creation of source colony

A total of 61 minor workers were obtained from the six colonies (Table 2). In two of the colonies, the

179 virus was not detected in any of the workers analyzed. In the other four colonies, the virus was detected
180 in 91% to 100% of the workers.
181
182 4.2 Fostering experiment
183 In two experimental colonies with CYV-uninfected nurse workers, a total of 61 eggs were introduced
184 from CYV-infected source colonies (Table 3). Fifteen workers emerged from among them, most of
185 which are CYV positive. CYV-negative workers remained non-infected after caring for infected eggs
186 through to emerge in adults.
187 A total of 49 eggs were introduced from CYV-uninfected source colonies into two experimental
188 colonies with CYV-infected nurse workers, and 8 adults emerged. None of these individuals were
189 infected with the virus (Table 3).
190
191 **Discussion**
192 Hosts with unique ecologies and viruses may establish distinctive relationships. This study examined
193 the characteristics of CYV that infect ants exhibiting polydomy and polygyny. CYV was detected in
194 all nests except for one of the host ant *Camponotus yamaokai*, and the prevalence of CYV at collection
195 sites was over 60% for all locations. There was no significant difference in CYV prevalence among
196 castes. Horizontal transmission of CYV among nestmates was not detected, suggesting that the main
197 transmission route is vertical. These results indicate that CYV is widely distributed across the habitat
198 range of *C. yamaokai* through vertical transmission.
199
200 **1. CYV prevalence**
201 *Camponotus yamaokai* has queens, alate females, and males as reproductive castes, and major and
202 minor workers as labor castes within the nest [16]. Each caste exhibits distinct morphology and
203 physiological mechanisms. Furthermore, the division of labor among individuals leads to differences
204 in pathogen exposure [17, 18]. Therefore, the susceptibility to CYV and prevalence might vary among
205 castes. However, no significant differences in CYV prevalence among castes were detected. In ants,
206 sex is determined by whether the egg is fertilized; females develop from fertilized eggs, while males
207 develop from unfertilized eggs through parthenogenesis [19]. Whether female eggs become workers
208 or queens is generally determined by environmental and social factors during development [20]. In
209 CYV-infected queens, virus particles were observed within oocytes [8], suggesting that the
210 transmission of CYV to eggs occurs independent of caste determination, assuming there is no vertical
211 transmission from males. We did not test the vertical transmission from males. If vertical transmission
212 from males occurs, a difference in infection rates between the queens and the offspring would be
213 expected; however, such a difference was not detected. Therefore, without the mechanisms discussed
214 below, the vertical transmission from males either does not occur or is rare. Since *C. yamaokai* is

polygynous [21], the CYV prevalence in the colony is likely largely determined by the infection status of the queens, independent of the caste. Vertical transmission of CYV is likely to be related to the infection rate.

If vertically transmitted viruses reduce host fitness, non-infected individuals will be selected, causing the prevalence in the host population to decrease. If the virus does not affect host fitness (neutral), selection due to infection will not occur, and stochastic fluctuations will cause the prevalence in the host population to approach either 0% or 100% [22]. However, the wild population-level prevalence of CYV was over 60% at all collection sites. The following factors could explain the maintenance of such a prevalence. I) CYV may influence host traits, affecting the reproduction and survival of the colony. In eusocial insects, genetic diversity within the colony can enhance foraging efficiency and resistance to pathogens, thus increasing colony fitness [23, 24]. If CYV impacts host traits, the presence of both infected and uninfected individuals in the colony could produce effects similar to genetic diversity, potentially increasing colony fitness. Therefore, selective pressure may be acting to maintain the observed CYV prevalence in the wild. II） The vertical transmission rate of CYV from infected queens to eggs is not 100%, meaning that even if the queen is infected, some of the next generation remain uninfected. In intraspecific fostering experiments, some source colonies showed CYV prevalence of about 90%. This suggests that vertical transmission of CYV was not observed in some individuals. Because some of the next generation of queens remain uninfected, a certain proportion of uninfected hosts are maintained. In this case, to sustain the infection, some mechanisms, such as vertical transmission from males, are needed to compensate for the decrease in prevalence caused by such incomplete vertical transmission from queens. Otherwise, the prevalence of CYV would quickly drop to 0%. It should be noted that the vertical transmission rate in the fostering experiments may be influenced by the experimental treatments.

**2. CYV transmission pathways**

CYV was suggested to undergo vertical transmission, but horizontal transmission has not been verified. In intrapecific rearing experiments, horizontal transmission of CYV through trophallaxis between adult and larval *C. yamaokai* was not detected. In addition, in these experiments, the number of emerged workers was low relative to the number of eggs introduced to the experimental colonies. Many of the broods were likely consumed by adult ants; however, CYV was not detected in the nurse workers. These results suggest that horizontal transmission of CYV through food intake including trophallaxis is rare, if any. Although the possibility of horizontal transmission of CYV between adults has not been tested, trophallaxis in ants of the subfamily Formicinae is frequent and occurs among all colony members, including between adults and larvae, and among adults[25]. Therefore, if horizontal transmission were to occur, it is expected that the prevalence among adults would quickly reach 100%. However, the prevalence in the wild remains around 80%, suggesting that horizontal transmission

among adults is also either non-existent or rare, like that between adults and larvae. Furthermore, the results that CYV was detected in adult *C. yamaokai* reared by non-infected *C. nawai* support the notion that horizontal transmission is not necessary for maintaining CYV infection.

Virus particles were observed within the cytoplasm of oocytes [8], suggesting that these particles serve as a source of infection for the next generation of host ants. Parasites that rely primarily on vertical transmission tend to reduce their pathogenicity through coevolution with their hosts [26]. No clear pathology has been detected in ants infected with CYV, suggesting that CYV may have evolved to reduce or lose its pathogenicity through vertical transmission. However, for viruses with biparental vertical transmission from males and females, even with increased pathogenicity, the infection can still be sustained, unlike viruses that rely solely on vertical transmission from females [27–29].

## 3. CYV distribution

To understand the relationship between CYV and its host ants, we examined the geographical distribution of CYV in Japan. The host ant, *C. yamaokai*, is endemic to Japan and is distributed from Kyushu to Tohoku [16], and we collected the ants from seven locations from the host distribution across Japan. We detected CYV in all seven locations in this study. This suggests that the distribution of the host ant and CYV coincides. Since CYV was not detected in closely related species of *Camponotus* [8], and vertical transmission is primary route of CYV infection, the initial population of the host ant might be infected with CYV early in its speciation process. As the infected *C. yamaokai* expanded its distribution across Japan, CYV's distribution expanded as well.

## 4. Conclusion

In this study, we examined the characteristics of the toti-like virus infecting ants with unique ecology. We found no caste differences in the virus infection rate, and most colonies exhibited a substantially high infection rate, suggesting that CYV infects the host before the caste is determined. Furthermore, the geographical distribution and transmission pathways of CYV indicate that it expanded its distribution along with the host's spread, primarily due to vertical transmission. The mechanisms by which CYV is maintained within the host population remain to be elucidated in future studies.

**Acknowledgment**

We would like to express our sincere gratitude to Professor Toshiyuki Fukuhara for granting us access to the analytical equipment, which greatly contributed to this research.

We would like to express our gratitude to Tatsuya Ishizuka, Takuto Hashizume, Kohei Nakatsuji, Tsubasa Nozaki, Atushi Saito, Ryota Seki, Soki Shinkawa, Yu Takatani, and Hiro Yoshimura for their assistance in the field. We also thank the staff of the Shiiba Research Forest of Kyushu University for their support in conducting this research.

**Funding information**

This work was supported by JSPS KAKENHI Grant Numbers JP20K22648, JP24K08932.

**Conflict of Interest**

Authors declare no conflict of interest.

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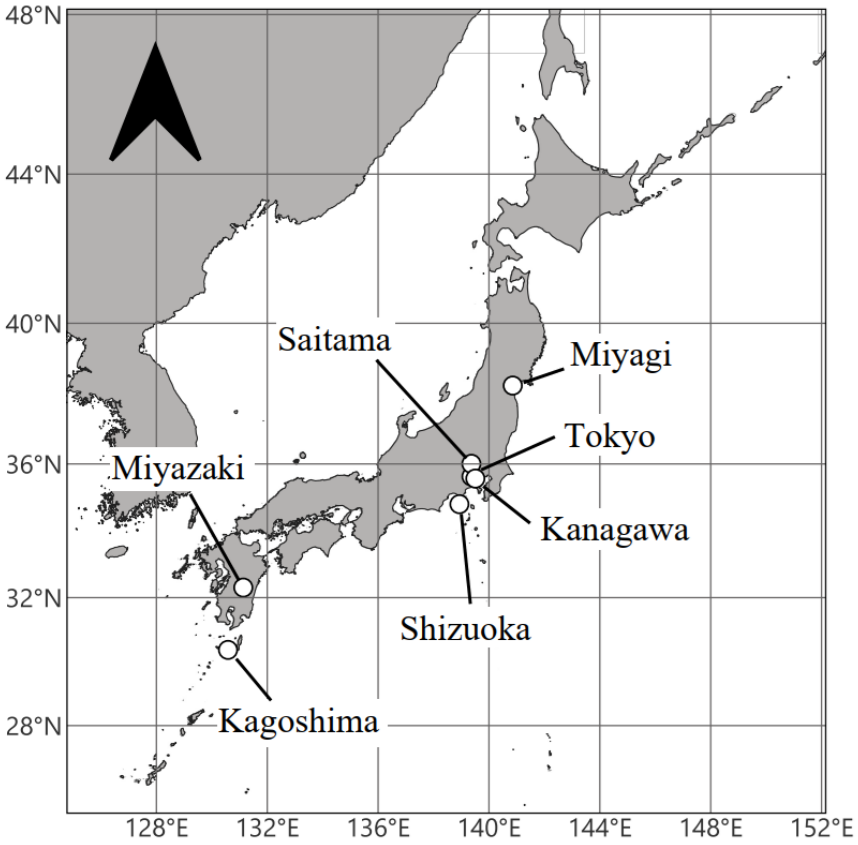


379

380 **Figure 1** Collection locations of *Camponotus yamaokai*.

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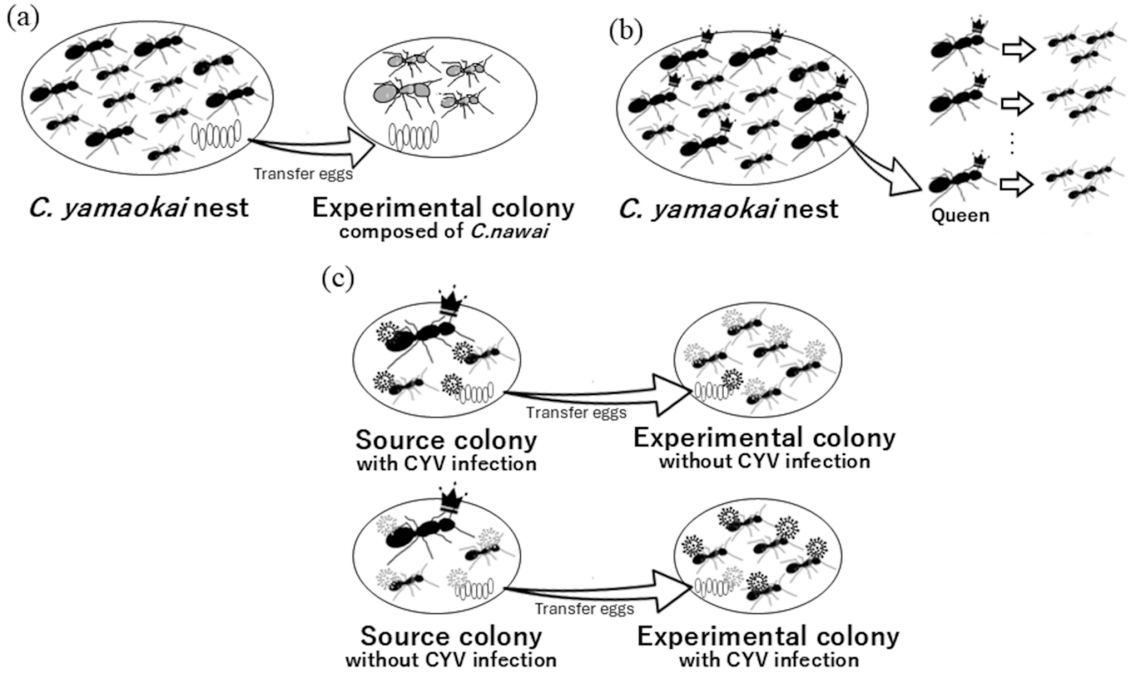


**Figure 2** Diagrams of interspecific and intraspecific fostering experiments. (a) Interspecific fostering experiment. This experiment examined whether CYV is transmitted vertically through reproduction. (b) Creation of source colony. Each source colony consists of the offspring of a single queen. This experiment was conducted to confirm that CYV infection remained consistent within the source colony. (c) Intraspecific fostering experiment. This experiment examined whether CYV is transmitted horizontally through trophallaxis.

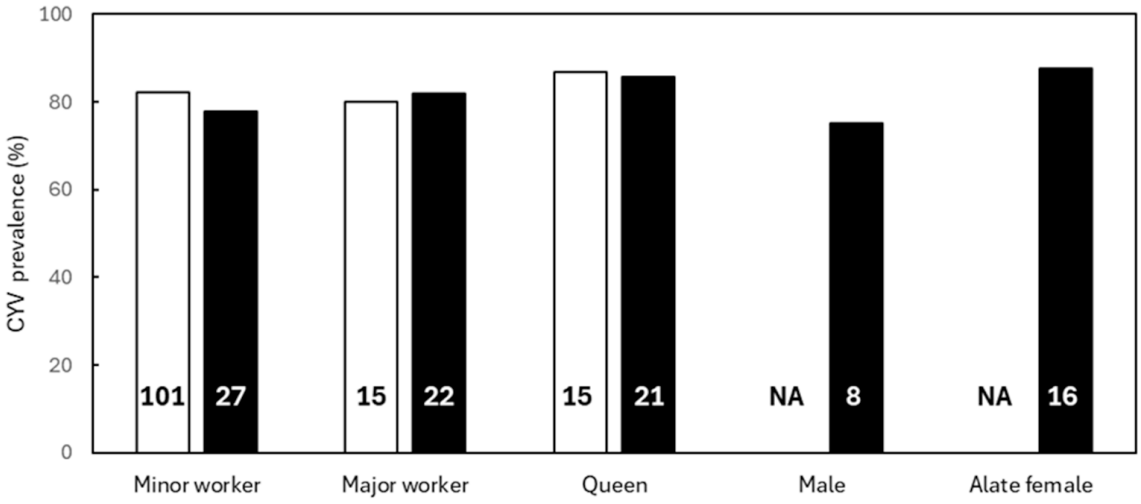


**Figure 3** CYV prevalence of each caste. The white bars indicate the prevalence in Tokyo, while the black bars indicate that in Kanagawa. The numbers within the bars represent the sample size.

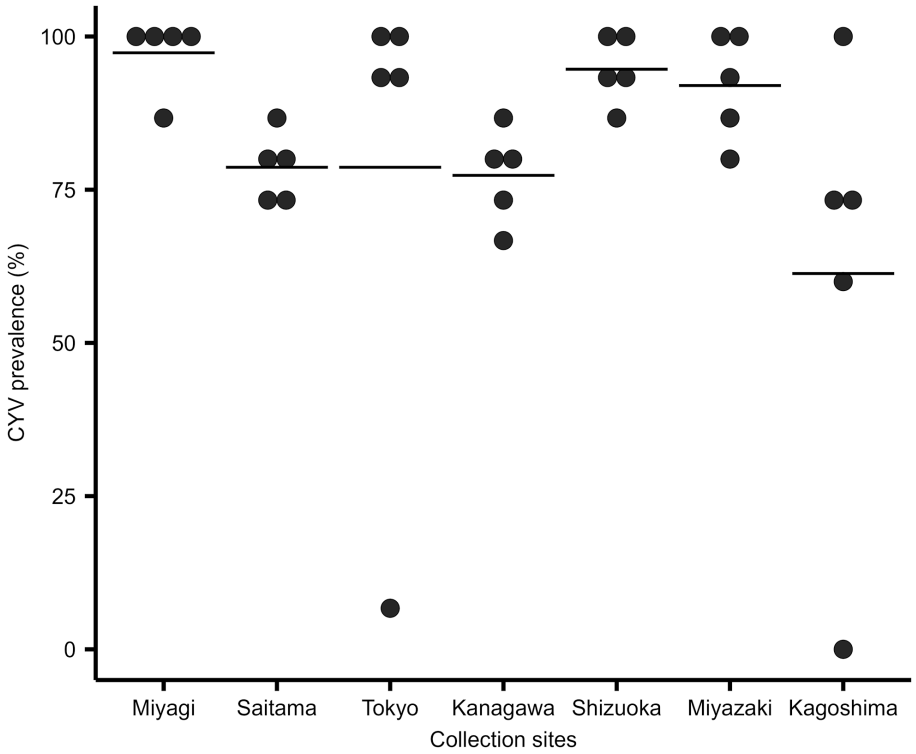


**Figure 4** CYV prevalence at seven locations. Intra-colonial CYV prevalence was assessed in five nests per location. Fifteen minor workers from each colony were analyzed individually. Each point represents the prevalence within a single nest, while the bars indicate the average prevalence across the five nests in each location.

**Table 1** Infection status of *C. yamaokai* reared by *C. nawai*. Eggs of *C. yamaokai* were reared by workers of *C. nawai* that were not infected with CYV. The individuals that grew to adulthood were used for the investigation of CYV infection rates.

| Eggs introduced | Workers emerged | CYV positive (%) |
|---|---|---|
| 85 | 0 | NA |
| 71 | 4 | 3 (75) |
| 111 | 8 | 6 (75) |

404 **Table 2** The CYV infection of the offspring workers of a single queen.

| Colony ID | n | CYV positive (%) |
|---|---|---|
| 1 | 9 | 0 (0) |
| 2 | 9 | 0 (0) |
| 3 | 8 | 8 (100) |
| 4 | 11 | 8 (91) |
| 5 | 12 | 11 (92) |
| 6 | 12 | 12 (100) |

405

406 **Table 3** Infection status of *C. yamaokai* reared by individuals of the same species with different
407 CYV infection profiles. Infection status of the source colonies was determined before the fostering
408 experiment. The eggs of *C. yamaokai* were reared by workers with different CYV infection statuses.
409 The individuals that grew to adulthood were used for the investigation of CYV infection rates.

| Infection status of the source colony | | Eggs introduced | Emerged worker | | CYV prevalence of nurse workers |
|---|---|---|---|---|---|
| Eggs | Nurse workers | | n | CYV positive (%) | |
| Positive | Negative | 21 | 5 | 5 (100) | 0% |
| | | 40 | 10 | 7 (70) | 0% |
| Negative | Positive | 10 | 3 | 0 (0) | 90% |
| | | 39 | 5 | 0 (0) | 80% |

410