Mate discrimination of *Colocasiomyia xenalocasiae* and *C. alocasiae* (Diptera: Drosophilidae) as a possible factor contributing to their co-existence on the same host plant

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Abstract (150 to 250 words)

Mate discrimination contributes to the co-existence of related species by reducing the risk of interspecific copulation. In pollination mutualistic systems where pollinators utilize host plants as mating places, sharing of host plants with other related species could increase non-adaptive interspecific copulation. Although such host-sharing species are expected to have strong mate discrimination systems, little is known about whether and how they discriminate species for mating. Here, we investigate mate discrimination of two fly species, *Colocasiomyia xenalocasiae* and *C. alocasiae* (Diptera: Drosophilidae), which share host plants; they are essentially anthophilous, depending exclusively on specific aroid host plants throughout their entire life cycles. Our field observations showed that the males of *C. alocasiae* and *C. xenalocasiae* preferentially paired with conspecific, but not heterospecific, females. This indicates that they discriminate species for mating in the natural habitat. Such mate discrimination was also observed under laboratory conditions. To investigate how these flies discriminate species, we defined distinct behavioral elements in courtship sequence in both species, and compared sexual interactions in each element between conspecific and heterospecific pairs. We found that males discriminate female whilst tapping, whereas females discriminate male before or during males' attempted mounting. This suggests that mate discrimination systems in both males and females reduce the incidence of heterospecific mounting; mounting is a necessary step in the sequence of courtship for successful copulation. The mate discrimination system found in this study potentially allows for the co-existence of *C. xenalocasiae* and *C. alocasiae* on the same host plant by effectively suppressing interspecific copulation.

Introduction

Mate discrimination is essential to maintain the co-existence of closely related species by avoiding fitness reductions due to interspecific copulation (Noor 1999). In pairs of related species that co-habit in the same place as a mating site, the risk of non-adaptive interspecific copulation could increase. In such species pairs, mate discrimination is expected to be essential to reduce the risk of interspecific copulation (Friberg et al. 2008).

Many *Colocasiomyia* fly species (Diptera: Drosophilidae) co-exist, or co-habit, with closely-related species in their host plants, which mainly belong to the subfamily Aroideae (Sultana et al. 2006; Takano-Takenaka et al. 2021). Such intimate sharing of the same host plant by a pair of closely related species, a particular mode of pollination mutualism, is often reported in this genus, although some *Colocasiomyia* species monopolize their host plants (reviewed in Takano-Takenaka et al. 2021). The fly's life cycle completely depends on the host plants; they grow, feed, mate, and lay eggs in the host inflorescences. The host plants also depend on the flies for pollination (Yafuso 1993; Miyake and Yafuso 2003, 2005; Takano-Takenaka et al. 2012). Such brood-site pollination mutualism might be a major factor for the diversification of *Colocasiomyia* species and their host plants (*c.f.* Sakai 2002).

In southern Japan, *Colocasiomyia xenalocasiae* co-exists with *C. alocasiae*, both of which belong to the *Colocasiomyia cristata* species group, on inflorescences of *Alocasia odora* (Araceae) (Yafuso 1983, 1993). Previous studies suggest that this host-plant sharing was achieved by the ancestors of the *cristata* and *colocasiae* species subgroups, to which *C. xenalocasiae* and *C. alocasiae* belong respectively (Okada 1980; Takano-Takenaka et al. 2021). Larvae of *C. xenalocasiae* and *C. alocasiae* feed on different parts of the host inflorescences and infructescences, resulting in micro-allopatric breeding niche separation which allows for the co-existence of these two species on the same host plant (Yafuso 1994). On the other hand, adults of these two species assemble together and are collected simultaneously from the same inflorescence (Fig. 1) (Miyake and Yafuso 2005). Temporal and spatial habitat isolation of the adults has not been reported so far. It is predicted that they possess a mate discrimination system to prevent non-adaptive interspecific copulation. However, whether and how they discriminate mates from each other has not been investigated.

In this study, we performed field observations to examine whether *C. xenalocasiae* and *C. alocasiae* discriminate each other for mating in the wild. Next, by conducting laboratory observations, we first described behavioral elements in the courtship sequence. Furthermore, by comparing courtship sequences between conspecific and heterospecific pairs of these species, we infer how these related species discriminate mates from each other.

Materials and methods

Field observations

Field observations were performed on the Nishihara Campus of University of the Ryukyus (26°14'51.3"N 127°45'54.5"E) in Okinawa Main Island, Japan from April to June 2021. Coupling (i.e., a fly mounting another fly) pairs of *Colocasiomyia* flies were carefully aspirated from inflorescences of *A. odora*. Collected pairs were maintained separately for each pair and the species of each male and female was identified under a microscope using ice anesthesia based on the bristles of the costal vein (Okada 1975; 1980). In total 28 pairs were collected from four inflorescences of *A. odora*. Field collection of these species is not prohibited in the sampling area.

Fly maintenance under laboratory conditions

In addition to the coupling pairs, other adult flies of *Colocasiomyia* were collected from inflorescences of *A. odora* on the Campus from April to June 2021 and April 2022. After collection, the living flies were transferred to the laboratory in Nagoya University, Japan, identified their species and sexes, and maintained using *Drosophila* standard yeast-based media at 25°C in 40% to 60% relative humidity under a 12-h light/dark (LD) cycle. Males of *C. xenalocasiae* and *C. alocasiae* were maintained individually under isolated conditions. Females of *C. xenalocasiae* and *C. alocasiae* were maintained in a group of 10-20 individuals. These field-collected flies were used for video recording 5-10 days after the collection.

Video recording of mating behaviors

To describe the behavioral elements during courtship sequences (Fig. 2), a pair of male and female of *C. xenalocasiae* or *C. alocasiae* was introduced into each acrylic artificial chamber (diameter 1 cm, height 4 mm) and observed with the aid of video recording. To examine which behavioral elements of courtship contribute to mate discrimination (Figs 3-6), two males and two females were introduced and recorded in the same chambers. For the conspecific pairing condition, two *C. xenalocasiae* or *C. alocasiae* males were paired with two conspecific females. For the heterospecific paring condition, two *C. xenalocasiae* or *C. alocasiae* males were paired with two *C. alocasiae* or *C. xenalocasiae* females, respectively. Video recording, using a CMOS camera (DFK 33UP1300, The Imaging Source Asia Co., Ltd) equipped with a zoom lens (MVL50M23, Thorlabs, Inc.), was started immediately after the introduction of flies into the chambers (diameter 1 cm, height 4 mm; bottom is coated with *Drosophila* standard yeast-based media) and lasted for 30 min with a resolution of 1280 x 1024 pixels at 30 fps. Behavioral assays were performed during the light phase of the LD cycle at 25°C and 40% to 60% relative humidity.

The numbers of chambers (i.e., replicates) for *C. xenalocasiae* males were 14 for conspecific pairs and 18 for heterospecific pairs. Those for *C. xenalocasiae* males were14 for conspecific pairs and 36 for heterospecific pairs.

Behavioral analysis

For the behavioral analysis, we defined the behavioral elements during the courtship sequence as follows:

Orientation: male directing his body axis toward a female when she was within one-body length from the male.

Tapping: male touching a female's body with his forelegs.

Attempted mounting: male turning behind a female and grasping her abdomen with his forelegs.

Mounting: male being on a female, grasping her thorax with his forelegs.

Wing lifting: female lifting both wings immediately after male's attempted mounting.

The mounting duration of males toward conspecific or heterospecific females was measured using the video annotation software vCode (Hagedorn et al. 2008) (https://social.cs.uiuc.edu/projects/vcode.html). The duration was defined as the length of time between when a male started to mount a female after her wing lift and when the male dismounted from the female or the end of video recordings (at 30 min). Because there are two males in a chamber, we used the mean value of mounting durations of the males as a representative value. The mounting index was then calculated for each chamber as the percentage of mounting duration to the 30 min observation period. The numbers of chambers for males having performed orientation, tapping, and attempted mounting were manually counted. We assigned the chamber as positive when at least one male in a chamber showed the corresponding behavior during the observation period. Then the incidence of each behavioral element was calculated by $\frac{N_{positive}}{N_{all}}$, where $N_{positive}$ and N_{all} are the number of positive chambers and all chambers, respectively. The numbers of chambers for females having performed wing lifting toward conspecific and heterospecific males were manually counted and calculated by $\frac{N_{positive}}{N_{all}}$ as described above. Here, we used the chambers in which males had performed attempted mounting for analysis. The numbers of chambers were as follows: For *C. xenalocasiae*, n = 14 chambers for conspecific pairs and n = 18 chambers for heterospecific pairs; for *C. alocasiae* males, n = 14 chambers for conspecific pairs and n = 36 chambers for heterospecific pairs (Figs 3, 4 and 6). For *C. xenalocasiae* females, n = 14 chambers for conspecific pairs and n = 5 chambers for heterospecific pairs; for *C. alocasiae* females, n = 6 chambers for conspecific pairs and n = 12 chambers for heterospecific pairs (Fig. 5).

Statistics

Statistical analysis was performed using the R software (version 4.0.3). Fisher's exact test for count data was used to compare the incidence ratios of chambers that contained flies having performed each behavioral element (*i.e.*, orientation, tapping, attempted mounting, wing lifting, and mounting). The Brunner-Munzel test was used for comparison of mounting index between conspecific and heterospecific pairs, after verifying the equality of variance and normality of the values by F-tests and Shapiro-Wilk tests, respectively. For the Brunner-Munzel test, the brunnermunzel package (version 1.4.1) was used (https://github.com/toshi-ara/brunnermunzel). Graphs were prepared using Prism7 (GraphPad Software Inc.).

Results

To examine whether *C. xenalocasiae* and *C. alocasiae* discriminate conspecifics as a mating partner, we observed their mating behavior in the field. In the wild population of these species, flies mounting other individuals were often observed (Fig. 1B), which was considered to be post-mounting male courtship because similar behavior is often observed in *Drosophila* species, including the *montium* species subgroup (Hoikkala et al. 2000; Chen et al. 2013, 2019). We collected 28 coupling pairs (i.e., a fly mounting another fly) of the *Colocasiomyia* flies from inside of *A. odora* inflorescences blooming in the wild and identified the sex and species. All pairs were comprised of a male and a female, implying the mounting is a part of mating behavior for these species. Seven out of 28 pairs were *C. xenalocasiae*, and the other 21 pairs were *C. alocasiae,* while no heterospecific pairs were found (Table 1). *Colocasiomyia xenalocasiae* and *C. alocasiae* were often collected from the same inflorescences (Table 1). These results indicate that both species discriminate conspecific mates for mating even when they co-exist in the same host inflorescences in the wild.

Flies discriminate species through courtship rituals (Coyne and Orr 1989; Yukilevich and Peterson 2019). However, how *Colocasiomyia* interacts with their potential mating partners during courtship has not been reported so far. Here, we first describe behavioral elements of the courtship sequence of *C. xenalocasiae* and *C. alocasiae* in the experimental condition with an artificial chamber (*C. xenalocasiae*: Fig. 2A-H; *C. alocasiae*: Fig. 2I-P). In both species, a male localized toward the female and tapped her with his forelegs when he encountered a female (orientation and tapping, respectively; Fig. 2A, B, I and J). These behavioral elements are commonly found in the courtship of other drosophilid species (Spieth 1952; Wen and Li 2011; Khallaf et al. 2021). After tapping, the male quickly turned behind the female and grasped her abdomen with his forelegs to attempt to mount (attempted mounting; Fig. 2C and K). Such direct transition from tapping to attempted mounting is relatively unique in Drosophilidae, as males of many drosophilid species show further pre-mounting courtship rituals after tapping, such as wing vibration to emit courtship songs, wing displaying, and/or licking female's genitalia (Spieth 1952; Wen and Li 2011; Khallaf et al. 2021). When males attempted to mount, females showed two types of behavior; one for acceptance and the other for rejection. In most cases, females showed acceptance by lifting both wings to enable the male to mount completely (wing lifting of female; Fig. 2D-F, L-N). In a few cases, however, females did not show this wing lifting, which led to the failure of the attempted mounting. As the failure of the attempted mounting resulted in the end of the sexual interaction (data not shown), this female behavioral choice is the major step to achieving copulation in these species.

After a successful mounting, the male occasionally displayed a quick scissoring of both wings during mounting (wing scissoring; Fig. 2G and O). In many drosophilid species, copulation starts simultaneously with the initiation of mounting (Massey et al. 2019). This is not the case, however, in *C. xenalocasiae* and *C. alocasiae* males; after 1-2 min from the start of mounting, males attempted to copulate by bending their abdomens (attempted copulation; Fig. 2H and P). In our observations, only a few cases of obvious copulation with genitalia coupling were observed. Occasionally, when males attempted to copulate by bending the abdomen, females extended their genitalia, which generally functions as rejection in many drosophilid (Wang et al. 2020). Since all attempted copulations were observed during mounting, mounting was thought to be an indispensable behavioral step for males to achieve successful copulation. Intriguingly, in licking, the male licks dorsal thorax of his partner during mounting, was observed in four out of 8 *C. alocasiae* males that showed mounting but zero out of 16 *C. xenalocasiae* males (Fig. 2O). This licking behavior is unique to this species, as that of many *Drosophila* species typically targets female genitalia to lick. Overall, our observations suggested that the sequence of courtship behavior is common in both species except the licking behavior (Fig. 2Q). We also found that the incidence of attempted mounting in *C. alocasiae* males was much smaller than that in *C. xenalocasiae* under our laboratory conditions (42.9% in *C. alocasiae*, 100% in *C. xenalocasiae*,). It is likely that *C. alocasiae* flies have lower mating motivation than *C. xenalocasiae*, or alternatively, our laboratory conditions were not optimal for *C. alocasiae* flies.

To verify whether *Colocasiomyia* males discriminate species under the experimental condition, we compared the mounting index, i.e., the relative duration of mounting to the total observation time. In *C. xenalocasiae*, the mounting index of conspecific pairs was approximately 50% on average, showing that males spent half of the observation time engaging in mounting females (Fig. 3A). In contrast, the average conspecific mounting index in *C. alocasiae* was approximately 7.2%, much less than that of *C. xenalocasiae* (Fig. 3B). The mounting index of heterospecific pairs in both species was nearly zero, indicating that successful mounting rarely occurred (Fig. 3). In both species, the mounting index was significantly larger in conspecific pairs than in heterospecific pairs (Brunner-Munzel Test; *C. xenalocasiae*: p = 2.2E-16, Statistical value = -Inf; *C. alocasiae*: p = 0.012, Statistical value = -2.8938) (Fig. 3). These results imply that both *C. xenalocasiae* and *C. alocasiae* males discriminate female species under the experimental condition without host plants.

To determine how the *Colocasiomyia* flies discriminate species, we compared the incidence ratio of each behavioral element during the courtship sequence between conspecific and heterospecific pairs. We quantified the ratios of the chambers (i.e., replicates) in which the flies showed each focal behavioral element (see Materials and Methods). For the two early-step behavioral elements, orientation and tapping, the incidence ratios of conspecific and heterospecific pairs were equally high and not significantly different (Fisher's exact test for count data; *C. xenalocasiae*: p = 1 in both orientation and tapping; *C. alocasiae*: p = 1 in both orientation and tapping) (Fig. 4A-D). This implies that males from either species do not discriminate species before orientation and tapping.

For attempt mounting, on the other hand, *C. xenalocasiae* males in conspecific pairs showed a significantly higher incidence ratio compared to those in heterospecific pairs, although approximately 60% of males attempted to mount heterospecific females (Fisher's exact test for count data; p = 0.024; Incidence ratio; conspecific pairs: 1, heterospecific pairs: 0.67) (Fig. 4E). In *C. alocasiae* males, the incidence ratio for attempted mounting showed a higher tendency in conspecific pairs than in heterospecific pairs, although no significant difference was detected (Fisher's exact test for count data; p = 0.15; Incidence ratio; conspecific pairs: 0.43, heterospecific pairs: 0.19) (Fig. 4F). These results suggest that *C. alocasiae* males partly discriminate species before the attempted mounting, but the discrimination was not as evident as in *C. xenalocasiae* males.

Even though some males in either species attempted to mount heterospecific females, almost no successful mounting occurred in heterospecific pairs (Fig. 3A and B). This suggests that females may also discriminate species. To test this idea, we compared female wing lifting to accept conspecific or heterospecific males' attempted mounting. To examine the acceptance of the females for whom mounting by a male had been attempted, we analyzed the chambers in which males had performed attempted mounting for the following analysis (*C. xenalocasiae* females; n = 14 chambers for conspecific pairs; n = 7 chambers for heterospecific pairs; *C. alocasiae* females; n = 6 chambers for conspecific pairs; n = 12 chambers for heterospecific pairs). In the conspecific pairs of both species, wing lifting of the females occurred in all chambers (Fig. 5A and B; but female rejection sometimes happens even when conspecific males attempt; see Fig. 2I). In the heterospecific pairs, in contrast, wing lifting occurred only once out of 5 chambers in *C. xenalocasiae* females and once out of 12 chambers in *C.*

alocasiae females (Fig. 5A and B). The incidence ratios of wing lifting were significantly lower in heterospecific pairs than in conspecific pairs in both species (Fig. 5A and B; Fisher's exact test for count data; *C. xenalocasiae*: p = 0.00013; *C. alocasiae*: p = 0.00037). These results indicate that females also discriminate species before or during attempted mounting.

To examine the effect of discrimination in both males and females on the successful mounting, we compared the incidence ratio of mounting between conspecific or heterospecific pairs. In *C. xenalocasiae*, mounting occurred in all the tested chambers for conspecific pairs but occurred only once out of 18 chambers in heterospecific pairs (Fig. 6A). In *C. alocasiae*, mounting occurred in six out of 14 chambers for conspecific pairs but only once out of 36 chambers for heterospecific pairs (Fig. 6B). The incidence ratios of successful mounting were significantly higher in conspecific pairs than in heterospecific pairs in both species (Fig. 6A and B; Fisher's exact test for count data； *C. xenalocasiae*: p = 3.2E-8; *C. alocasiae*: p = 1.6E-11). These findings correspond well with the differences in the mounting durations (Fig. 3). Taken together, these results suggest that both males and females contribute to mate discrimination to decrease interspecific copulation between *C. xenalocasiae* and *C. alocasiae*.

Discussion

In this study, we demonstrated that *C. xenalocasiae* and *C. alocasiae*, which share host plants with related species, discriminate between conspecific and heterospecific mates in the process of courtship. Mate discrimination by both males and females contributed to selective mounting to conspecifics, which is a necessary step to achieve copulation. The mate discrimination system, which we identified in this study, potentially allows the co-existence of these two species on the same host plant by reducing the risk of interspecific copulation. Some species in the *cristata* group monopolize their host plants without any co-habiting species, *e.g.*, *C. sulawesiana* on *A. macrorrhizos* (L.) G. Don in South Sulawesi (Okada and Yafuso 1989), *C. steudnerae* on *Steudnera colocasiifolia* (Araceae) in Yunnan, southern China (Takenaka et al. 2006), and *C. sabahana* on *A. scabriuscula* in Malaysian Borneo (Takano-Takenaka et al. 2021). Comparison of mate discrimination ability between monopolizing and host-sharing species will reveal how host-plant sharing enhances the mate discrimination system in *Colocasiomyia*.

In this study, we rarely observed genitalia coupling, a sign of successful copulation, even in conspecific pairs. A possible cause for this low copulation success could be the low level of sexual receptivity in females we tested. Because we used adult females of *C. xenalocasiae* and *C. alocasiae* collected from the field, their copulation experience, which generally reduces female sexual receptivity (Kubli 1992), was not controlled, even though we isolated them for 2-8 days before the experiments. Technical limitations of video recording might also lead to failure of observation of genitalia coupling. Especially in *C. alocasiae*, we observed a low incidence of male attempted mounting and female wing lifting (Fig. 4). Further mate-choice experiments by using virgin flies are needed to evaluate the actual contribution of mate discrimination to preventing interspecific copulation.

Colocasiomyia xenalocasiae and *C. alocasiae* males preferentially court to conspecific females at the step of attempted mounting, but not before orientation and tapping, suggesting that they discriminate species by tapping. Previous studies in *D. melanogaster* indicated that tapping functions to discriminate species via detecting cuticular hydrocarbons (CHCs) on the female body surface (Greenspan and Ferveur 2000; Seeholzer et al. 2018; Ahmed et al. 2019). As the CHCs play a pheromonal role in mate discrimination in many insects including *Drosophila* (Jallon and David 1987; Billeter et al. 2009; McKinney et al. 2015; Shahandeh et al. 2018), they are expected to be involved in mate discrimination by these *Colocasiomyia* species as well. An interesting future study in line with this speculation would be to investigate whether mate discrimination is affected by the ablation of the male forelegs, a possible sensory organ necessary for CHCs detection.

We found in our experiment that almost all tested *C. xenalocasiae* and *C. alocasiae* females accepted the attempted mounting of conspecific males, but rarely accepted heterospecific males, indicating females also discriminate species. In our observations, no prominent courtship behavioral elements, other than orientation and tapping, were observed in males before attempted mounting. These findings suggest that *C. xenalocasiae* and *C. alocasiae* females discriminate species by detecting something during males' attempted mounting. Contact chemosensation is one possible way for female's mate discrimination. During the males' attempted mounting, the females are physically in contact with males at the dorsal body parts. In *D. melanogaster*, sensilla for contact chemosensation on the wing margin of females are suggested to regulate females' sexual receptivity by sensing the chemicals derived from males (He et al. 2019). Evaluating whether sensilla on the female wing margin contributes to the discrimination of mate species will elucidate the sensory mechanism underlying female mate discrimination in *Colocasiomyia*.

Our observations demonstrated that during pre-mounting courtship *C. xenalocasiae* and *C. alocasiae* males do not show any behavioral elements, such as wing vibration to emit courtship songs, wing displaying, and/or licking female's genitalia, which are conserved in many other drosophilid species (Spieth 1952; Wen and Li 2011; Khallaf et al. 2021). Such simplification of pre-mounting courtship was also previously found in the *montium* species subgroup (Hoikkala et al. 2000; Chen et al. 2013, 2019). One possible ecological factor that leads to the simplification of pre-mounting courtship could be the risk of mate interception, as found in *D. prolongata* (Setoguchi et al. 2015). If competitor males are densely present around the target female, the risk of mate interception would increase. Because *C. xenalocasiae* and *C. alocasiae* live in a dense assembly within the host inflorescences (Fig. 1), they are expected to be at high risk of mate interception during courtship, which may potentially lead to simplification of pre-mounting courtship.

The future application of genetic tools to *Colocasiomyia* species will enable the dissection of neural circuit mechanisms underlying the mate discrimination system and the simplified courtship rituals. Recent advances in transgenic and genome editing have allowed us to develop a better understanding of the neural circuit mechanisms responsible for species differences in courtship behavior in several *Drosophila* species (Tanaka et al. 2017; Seeholzer et al. 2018; Ding et al. 2019). Comparison of neural circuit mechanisms of *Colocasiomyia* species with that of other fly species, such as *D.*

melanogaster, will move us towards a better understanding of the diversification of mate discrimination system and courtship rituals.

Figure legends

Fig. 1 Assembling and sexual behavior of *Colocasiomyia* flies in wild inflorescences of their host plant *Alocasia odora*

(A) Assembling of *Colocasiomyia* flies in an inflorescence. (B) A *Colocasiomyia* fly mounting another fly (indicated with black arrowheads) in an inflorescence. The right-side panels show magnified views of flies boxed by a dashed black line in the left-side one.

Fig. 2 Behavioral elements in the courtship sequence of *Colocasiomyia* flies

(A-H) *C. xenalocasiae*. (I-P) *C. alocasiae*. (A, I) Male orientation: A male orients himself towards a female. (B, J) Tapping: A male taps a female body with his forelegs. Black arrowhead indicates a tapping foreleg. (C, K) Attempted mounting: A male grasps the abdomen of a female with his forelegs. (D, L) Wing lifting: A female lifts both wings to accept the attempted mounting of a male. Black arrowheads indicate the lifted wings of a female. (E, F, M, N) Mounting: A male mounts on a female. (G, O) Wing scissoring: A male continuously opens and closes his wings during mounting. Black arrowheads indicate the male scissoring wings. *C. alocasiae* males show licking (white arrowhead). (H, P) Attempted copulation: A male attempts to copulate with a female by bending his abdomen during mounting. (Q) Schematic flow of the courtship sequence in *C. xenalocasiae* and *C. alocasiae*. White and grey boxes indicate male and female behaviors, respectively. Parentheses indicate the behavioral element observed only in *C. alocasiae*.

Fig. 3 Mate discrimination of *C. xenalocasiae* and *C. alocasiae* under laboratory conditions

(A) Mounting index of *C. xenalocasiae* in conspecific (49.0 ± 17.4; mean ± standard deviation (SD); $n = 14$) or heterospecific (0.0093 ± 0.038; mean ± SD; $n = 18$) pairs. (B) Mounting index of *C. alocasiae* in conspecific (7.2 ± 13.5; mean ± SD; $n = 14$) or heterospecific pairs (0.0084 ± 0.050; mean ± SD; $n = 36$). Columns with error bars represent the mean of each group with SD. Each dot indicates the mounting index of each chamber. ***$p < 0.001$; *$p < 0.05$ (Brunner-Munzel test).

Fig. 4 Orientation, tapping, and attempted mounting of males toward conspecific and heterospecific females

(A, B) Incidence ratios of orientation of *C. xenalocasiae* (A) or *C. alocasiae* (B) males. (C, D) Incidence ratios of tapping of *C. xenalocasiae* (C) or *C. alocasiae* (D) males. (E,

F) Incidence ratios of attempted mounting of *C. xenalocasiae* (E) or *C. alocasiae* (F) male. n = 14 (*C. xenalocasiae* males toward conspecific females), 18 (*C. xenalocasiae* males toward heterospecific *C. alocasiae* females), 14 (*C. alocasiae* males toward conspecific females), and 36 (*C. alocasiae* males toward heterospecific *C. xenalocasiae* females), respectively. *p < 0.05; ns, not significant (Fisher's exact test for count data).

Fig. 5 Female wing lifting toward conspecific and heterospecific males.
(A) Incidence ratio of wing lifting of *C. xenalocasiae* females toward conspecific (n = 14) or heterospecific (n = 7; *C. alocasiae*) males. (B) Incidence ratio of wing lifting of *C. alocasiae* females toward conspecific (n = 6) or heterospecific (n = 12; *C. xenalocasiae*) males. ***p < 0.001 (Fisher's exact test for count data).

Fig. 6 Mounting ratio in conspecific and heterospecific pairs.
(A) Incidence ratio of successful mounting of *C. xenalocasiae* in conspecific (n = 14) or heterospecific (n = 18) pairs (B) Incidence ratio of successful mounting of *C. alocasiae* males in conspecific (n = 14) or heterospecific (n = 36) pairs. ***p < 0.001 (Fisher's exact test for count data).

C. xen♂ / *C. xen*♀	*C. alo*♂ / *C. alo*♀	*C. xen*♂ / *C. alo*♀	*C. alo*♂ / *C. xen*♀
7 (7)	21 (12)	0	0

Table 1 Numbers of conspecific and heterospecific coupling pairs of *C. xenalocasiae* and *C. alocasiae* collected from wild inflorescences of *A. odora*. The number of pairs collected from two-species mixed assemblies is shown in parentheses.

Supplementary information 1 Mounting index of *C. xenalocasiae* in conspecific or heterospecific pairs in each chamber (14 chambers for conspecific pairs; 18 chambers for heterospecific pairs).

Supplementary information 2 Mounting index of *C. alocasiae* in conspecific or heterospecific pairs in each chamber (14 chambers for conspecific pairs; 36 chambers for heterospecific pairs).

Data accessibility

Data of mounding index in *C. xenalocasiae* and *C. alocasiae* in conspecific and heterospecific pairs are provided in the electronic supplementary information.

Statements and Declaration

Conflict of Interest

The authors declare that they have no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

Author contributions

RT, YI, and AK contributed to the study conception and design. Material preparation and data collection were performed by RT and HT. Analysis was performed by RT. The first draft of the manuscript was written by RT, YI, and AK. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declarations

The authors have no relevant financial or non-financial interests to disclose. The authors have no competing interests to declare that are relevant to the content of this article. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. The authors have no financial or proprietary interests in any material discussed in this article.

References

Ahmed OM, Avila-Herrera A, Tun KM, *et al* (2019) Evolution of mechanisms that control mating in *Drosophila* males. Cell Rep 27:2527-2536.e4.

Billeter JC, Atallah J, Krupp JJ, *et al* (2009) Specialized cells tag sexual and species identity in *Drosophila melanogaster*. Nature 461:987–991.

Chen A li, Chen C cheng, Katoh T, *et al* (2019) Evolution and diversity of the courtship repertoire in the *Drosophila montium* species group (Diptera: Drosophilidae). J Evol Biol 32:1124–1140.

Chen CC, Watada M, Miyake H, *et al* (2013) Courtship patterns in the *Drosophila montium* species subgroup: Repeated loss of precopulatory courtship? Zoolog Sci 30:1056–1062.

Coyne JA, Orr HA (1989) Patterns of speciation in *Drosophila*. Evolution 43:362–381.

Ding Y, Lillvis JL, Cande J, *et al* (2019) Neural evolution of context-dependent fly song. Curr Biol 29:1089-1099.e7.

Friberg M, Vongvanich N, Borg-Karlson AK, *et al* (2008) Female mate choice determines reproductive isolation between sympatric butterflies. Behav Ecol Sociobiol 62:873–886.

Greenspan RJ, Ferveur JF (2000) Courtship in *Drosophila*. Annu Rev Genet 34:205–232.

Hagedorn J, Hailpern J, Karahalios KG (2008) VCode and VData: illustrating a new framework for supporting the video annotation workflow. In: Working Conference on Advanced Visual Interfaces. Naples, Italy, pp 317–321

He Z, Luo Y, Shang X, *et al* (2019) Chemosensory sensilla of the *Drosophila* wing express a candidate ionotropic pheromone receptor. PLoS Biol 17:1–27.

Hoikkala A, Crossley S, Castillo-Melendez C (2000) Copulatory courtship in *Drosophila birchii* and *D. serrata*, species recognition and sexual selection. J Insect Behav 13:361–373.

Jallon J-M, David JR (1987) Variation in cuticular hydrocarbons among the eight species of the *Drosophila melanogaster* subgroup. Evolution 41:294-302.

Khallaf MA, Cui R, Weißflog J, *et al* (2021) Large-scale characterization of sex pheromone communication systems in *Drosophila*. Nat Commun 12:4165.

Kubli E (1992) My favorite molecule. The sex-peptide. BioEssays 14:779–784.

Massey JH, Chung D, Siwanowicz I, *et al* (2019) The *yellow* gene influences *Drosophila* male mating success through sex comb melanization. Elife 8:e49388.

McKinney RM, Vernier C, Ben-Shahar Y (2015) The neural basis for insect pheromonal communication. Curr Opin Insect Sci 12:86–92.

Miyake T, Yafuso M (2003) Floral scents affect reproductive success in fly-pollinated *Alocasia odora* (Araceae). Am J Bot 90:370–376.

Miyake T, Yafuso M (2005) Pollination of *Alocasia cucullata* (Araceae) by two *Colocasiomyia* flies known to be specific pollinators for *Alocasia odora*. Plant Species Biol 20:201–208.

Noor MAF (1999) Reinforcement and other consequences of sympatry. Heredity (Edinb) 83:503–508.

Okada T (1980) Synhospitalic evolution of the genus *Drosophilella* Duda (Diptera. Drosophilidae), with description of a new species from Okinawa and Taiwan. Kontyu 48:218-225.

Okada T (1975) The oriental drosophilids breeding with flowers. Kontyu 43:356–363

Okada T, Yafuso M (1989) The genus *Colocasiomyia* Duda (Diptera, Drosophilidae) from Sulawesi. Proc Japanese Soc Syst Zool 39:48–55

Sakai S (2002) A review of brood-site pollination mutualism: Plants providing breeding sites for their pollinators. J Plant Res 115:161–168.

Seeholzer LF, Seppo M, Stern DL, Ruta V (2018) Evolution of a central neural circuit underlies *Drosophila* mate preferences. Nature 559:564–569.

Setoguchi S, Kudo A, Takanashi T, *et al* (2015) Social context-dependent modification of courtship behaviour in *Drosophila prolongata*. Proc R Soc B Biol Sci 282:20151377.

Shahandeh MP, Pischedda A, Turner TL (2018) Male mate choice via cuticular hydrocarbon pheromones drives reproductive isolation between *Drosophila* species. Evolution 72:123–135.

Spieth HT (1952) Mating behavior within the genus *Drosophila* (Diptera). Bulletin of the AMNH; v. 99, article 7. 9:

Sultana F, Hu YG, Toda MJ, *et al* (2006) Phylogeny and classification of *Colocasiomyia* (Diptera, Drosophilidae), and its evolution of pollination mutualism with aroid plants. Syst Entomol 31:684–702.

Takano-Takenaka K, Gao J, Hu Y, *et al* (2021) Phylogeny, taxonomy and flower-breeding ecology of the *Colocasiomyia cristata* species group (Diptera: Drosophilidae), with descriptions of ten new species. Zootaxa 5079:1–70

Takano-Takenaka K, Repin R, Mohamed MB, Toda MJ (2012) Pollination mutualism between *Alocasia macrorrhizos* (Araceae) and two taxonomically undescribed *Colocasiomyia* species (Diptera: Drosophilidae) in Sabah, Borneo. Plant Biol 14:555–564.

Takenaka K, Yin JT, Wen SY, Toda MJ (2006) Pollination mutualism between a new species of the genus *Colocasiomyia* de Meijere (Diptera: Drosophilidae) and *Steudnera colocasiifolia* (Araceae) in Yunnan, China. Entomol Sci 9:79–91.

Tanaka R, Higuchi T, Kohatsu S, *et al* (2017) Optogenetic activation of the *fruitless*-labeled circuitry in *Drosophila subobscura* males induces mating motor acts. J Neurosci 37:11662–11674.

Wang F, Wang K, Forknall N, *et al* (2020) Circuit and behavioral mechanisms of sexual rejection by *Drosophila* females. Curr Biol 30:3749-3760.e3.

Wen S-Y, Li Y-F (2011) An evolutionary view on courtship behavior of *Drosophila* : From a comparative approach. Low Temp Sci 69:87–100.

Yafuso M (1993) Thermogenesis of *Alocasia odora* (Araceae) and the role of *Colocasiomyia* flies (Diptera: Drosophilidae) as cross-pollinators. Environ Entomol 22:601–606.

Yafuso M (1983) Interspecific relationship between synhospitalic *Drosophilella* species (Diptera, Drosophilidae) inhabiting *Alocasia odora* on Okinawa Is., Japan. Kontyu 51:520–527

Yafuso M (1994) Life history traits related to resource partitioning between synhospitalic species of *Colocasiomyia* (Diptera, Drosophilidae) breeding in inflorescences of *Alocasia odora* (Araceae). Ecol Entomol 19:65–73.

Yukilevich R, Peterson EK (2019) The evolution of male and female mating preferences in *Drosophila* speciation. Evolution 73:1759–1773.

Fig.1

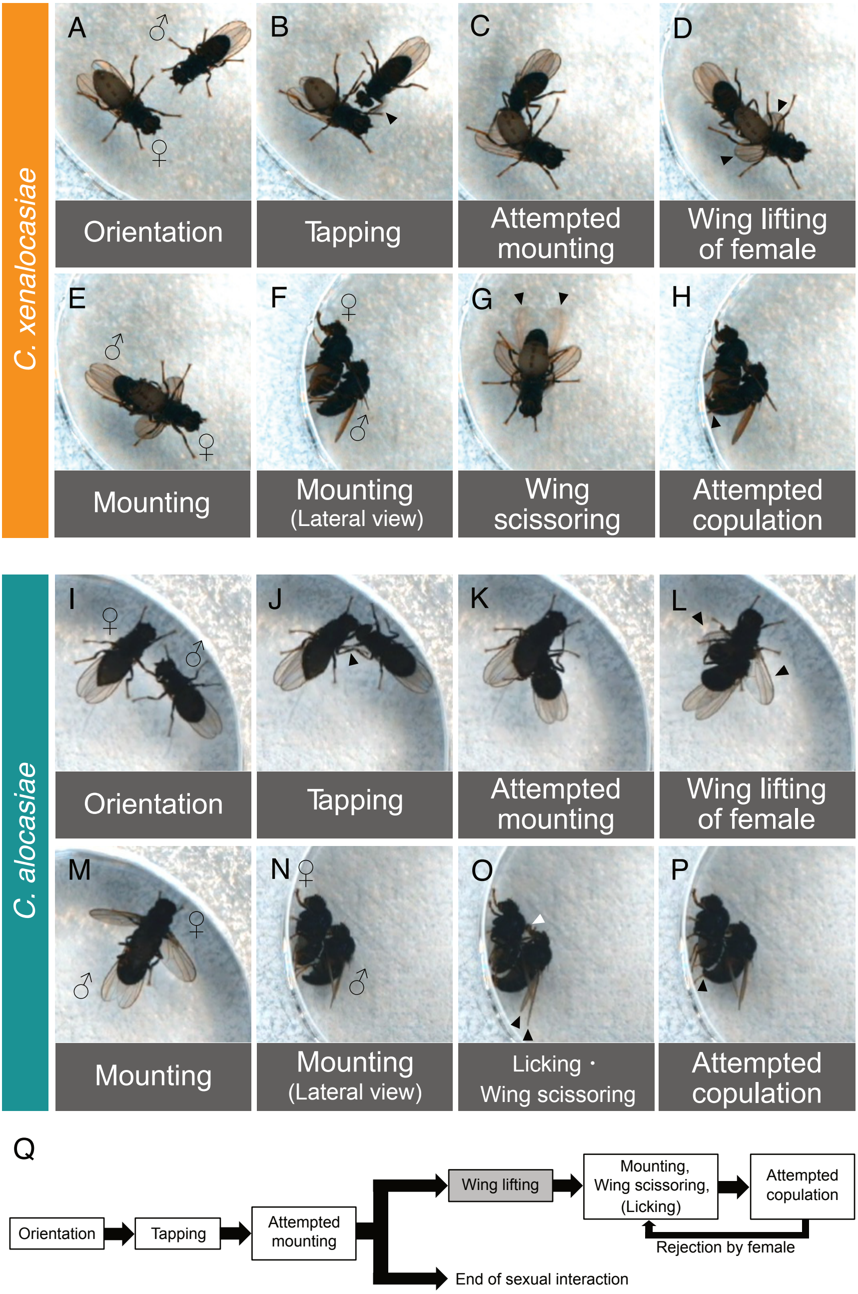
C. xenalocasiae
A ♂ ♀ Orientation
B Tapping
C Attempted mounting
D Wing lifting of female
E ♂ ♀ Mounting
F ♀ ♂ Mounting (Lateral view)
G Wing scissoring
H Attempted copulation
C. alocasiae
I ♀ ♂ Orientation
J Tapping
K Attempted mounting
L Wing lifting of female
M ♀ ♂ Mounting
N ♀ ♂ Mounting (Lateral view)
O Licking・Wing scissoring
P Attempted copulation
Q
Orientation
Tapping
Attempted mounting
Wing lifting
Mounting, Wing scissoring, (Licking)
Attempted copulation
Rejection by female
End of sexual interaction

Fig.2

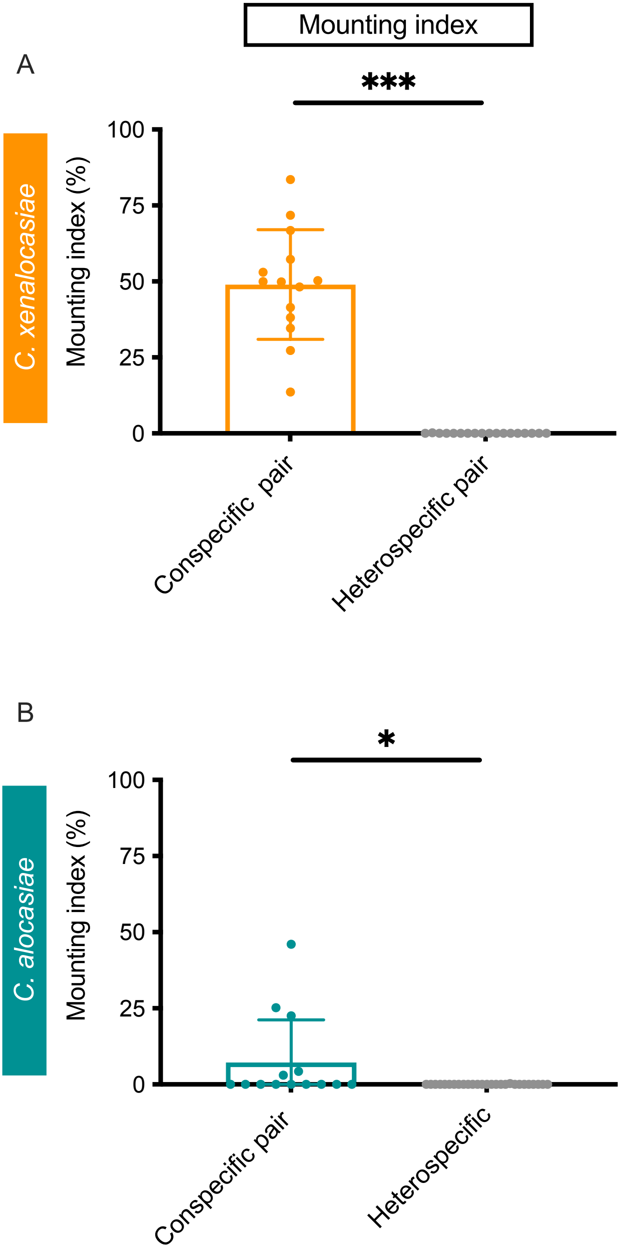
Mounting index
A
***
C. xenalocasiae
Mounting index (%)
100
75
50
25
0
Conspecific pair
Heterospecific pair
B
*
C. alocasiae
Mounting index (%)
100
75
50
25
0
Conspecific pair
Heterospecific

Fig.3

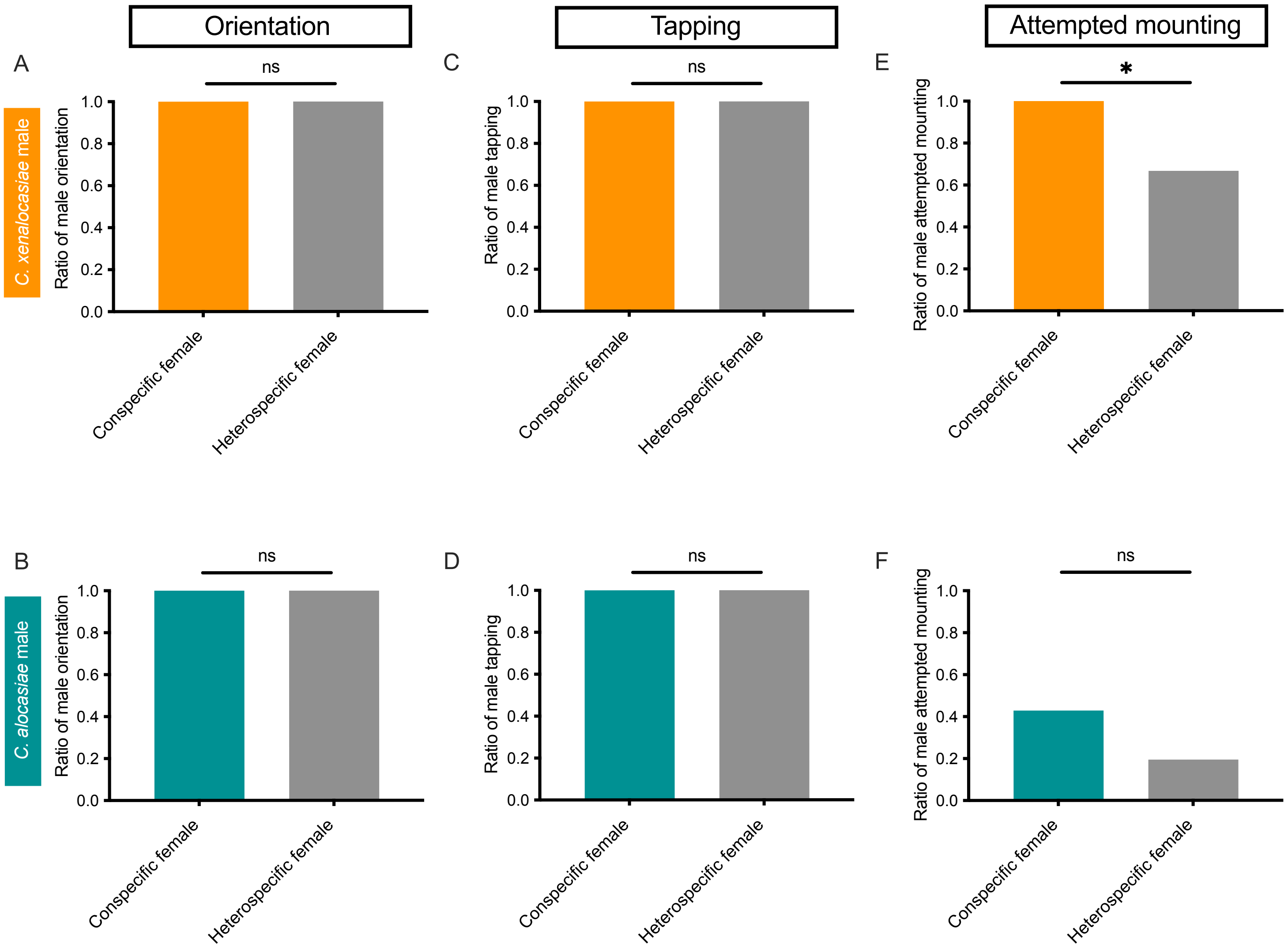
Orientation
Tapping
Attempted mounting
C. xenalocasiae male
C. alocasiae male
A
B
C
D
E
F
ns
ns
ns
ns
*
ns
Ratio of male orientation
Ratio of male tapping
Ratio of male attempted mounting
1.0
0.8
0.6
0.4
0.2
0.0
Conspecific female
Heterospecific female

Fig.4

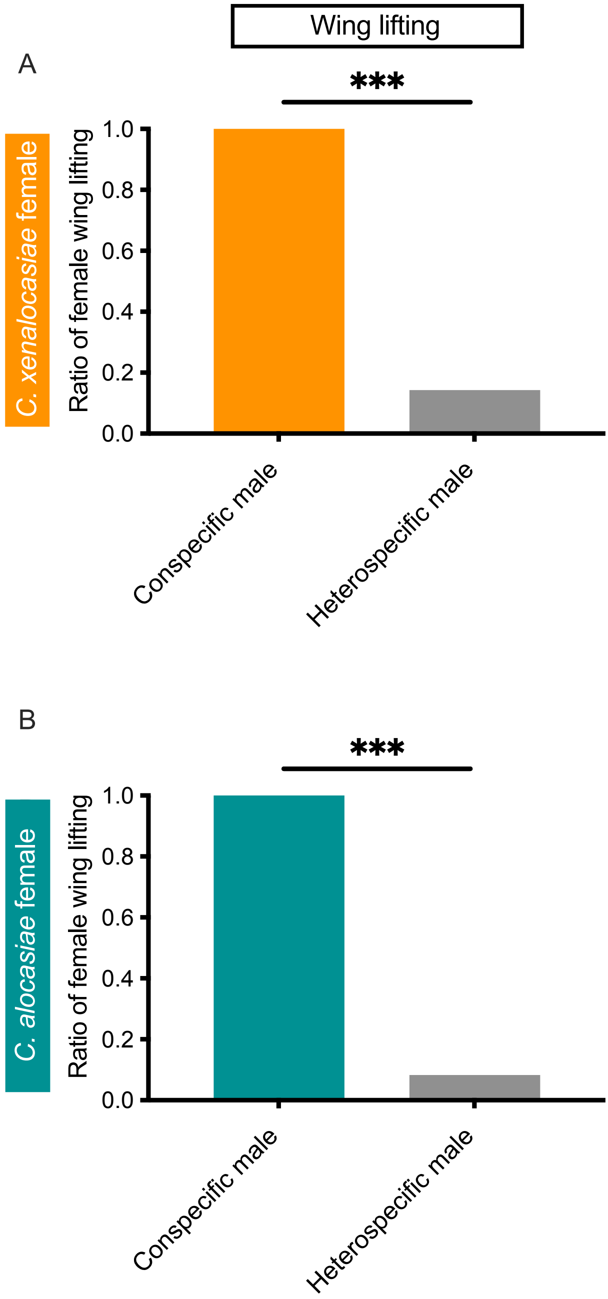
Wing lifting
A
***
C. xenalocasiae female
Ratio of female wing lifting
1.0
0.8
0.6
0.4
0.2
0.0
Conspecific male
Heterospecific male
B
***
C. alocasiae female
Ratio of female wing lifting
1.0
0.8
0.6
0.4
0.2
0.0
Conspecific male
Heterospecific male

Fig.5

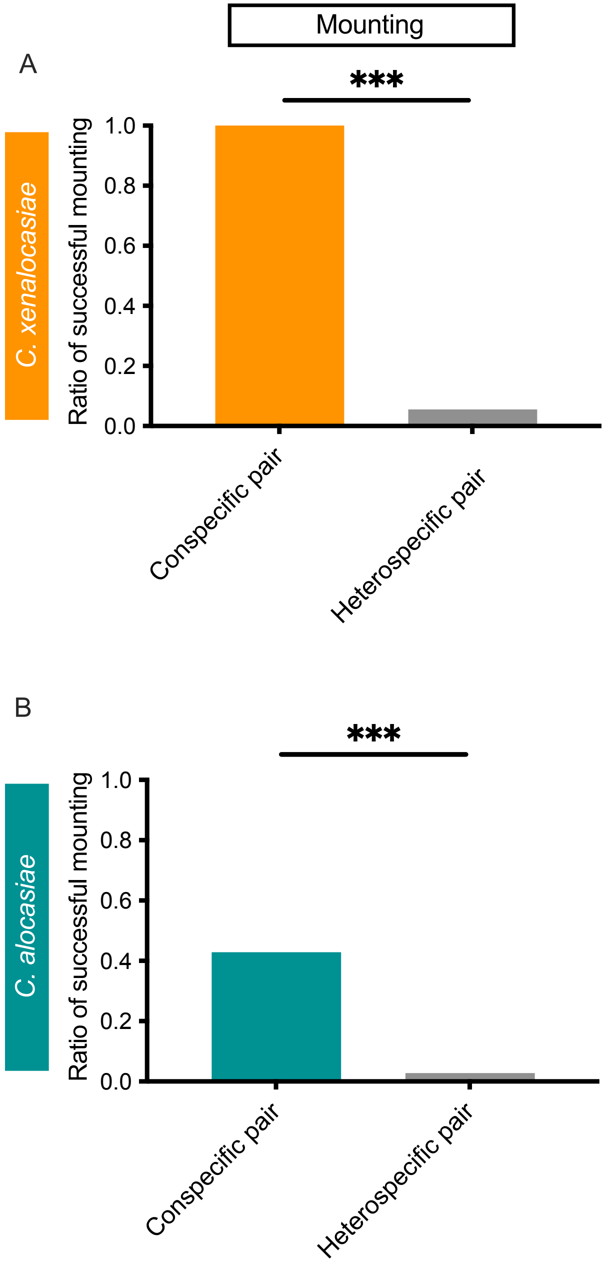
Mounting
A
***
C. xenalocasiae
Ratio of successful mounting
1.0
0.8
0.6
0.4
0.2
0.0
Conspecific pair
Heterospecific pair
B
***
C. alocasiae
Ratio of successful mounting
1.0
0.8
0.6
0.4
0.2
0.0
Conspecific pair
Heterospecific pair

Fig.6