

ARTICLE



Hybrids of two destructive subterranean termites established in the field, revealing a potential for gene flow between species

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Hybridization between invasive pest species may lead to significant genetic and economic impacts that require close monitoring. The two most invasive and destructive termite species worldwide, *Coptotermes formosanus* Shiraki and *Coptotermes gestroi* (Wasmann), have the potential for hybridization in the field. A three-year field survey conducted during the dispersal flight season of *Coptotermes* in Taiwan identified alates with atypical morphology, which were confirmed as hybrids of the two *Coptotermes* species using microsatellite and mitochondrial analyses. Out of 27,601 alates collected over three years, 4.4% were confirmed as hybrid alates, and some advanced hybrids (>F1 generations) were identified. The hybrid alates had a dispersal flight season that overlapped with the two parental species 13 out of 15 times. Most of the hybrid alates were females, implying that mating opportunities beyond F1 may primarily be possible through female hybrids. However, the incipient colony growth results from all potential mating combinations suggest that only backcross colonies with hybrid males could sometimes lead to brood development. The observed asymmetrical viability and fertility of hybrid alates may critically reduce the probability of advanced-hybrid colonies being established in the field.

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INTRODUCTION

Human activities such as urbanization, industrialization, and global trade have significantly altered habitats and distributions for many species. The spread of invasive organisms can lead two species that evolved in separate geographic areas to hybridize (Chunco 2014; Crispo et al. 2011). This kind of human-mediated hybridization has been observed in various organisms, e.g., plants, insects, fish, reptiles, birds, and mammals (Barbanera et al. 2010; Fitzpatrick and Shaffer 2007; Mattucci et al. 2019; Ohadi et al. 2016; Rubidge et al. 2001; Scott Schneider et al. 2004), and also in social insects like termites, bumblebees, bees, and ants (Calfee et al. 2020; Cohen and Privman 2019; Kanbe et al. 2008; Lefebvre et al. 2008).

Hybridization can create novel genome combinations that often break the long-time coordination of gene linkages. On the one hand, when hybrid offspring are unfit, it can lead to outbreeding depression (Frankham et al. 2011; Price and Waser 1979), a condition where genetic incompatibility results in poor health or lower reproductive success, which, in theory should reinforce reproductive isolation between parental species (Liou and Price 1994; Servedio and Noor 2003). On the other hand, when hybrid organisms are viable and fertile, it can lead to genomic admixture or gene introgression within parental species populations (Arnold et al. 1999; Slatkin 1985; Wright 1931). This means that hybridization can have long-term impacts on population genetic structure and is now considered a major force of diversification for evolution to act upon (Moran et al. 2021; Stebbins 1959).

Species closely related to human society are highly susceptible to hybridization effects. For instance, Spanish and German populations of mice *Mus musculus domesticus* hybridized with *M. spretus* from Algeria, introducing a rodenticide resistance gene into the *M. m. domesticus* genome, enabling resistance to warfarin-based rodenticides (Song et al. 2011). In South America, hybridization between native cotton bollworm (*Helicoverpa zea*) and introduced corn earworm (*Helicoverpa armigera*) led to *H. zea* acquiring pesticide resistance genes from *H. armigera* (Valencia-Montoya et al. 2020). Globally spreading pigs and bees in animal husbandry promote hybridization through natural processes or artificial selection (Calfee et al. 2020; Goedbloed et al. 2013). Invasive feral swine cause issues like crop damage, disease spread, and ecological disruption (Bevins et al. 2014). Africanized honey bees pose challenges to public health and beekeeping (Schumacher and Egen 1995). This evidence illustrates how genetic variation enhances hybrid populations' resilience to environmental challenges (Ellstrand and Schierenbeck 2000; Rieseberg et al. 2007), highlighting the intriguing interplay of human activity and hybridization events.

Coptotermes formosanus and *C. gestroi*, economically significant and invasive subterranean termite pests (Su 2002; Rust and Su 2012), evolved in allopatry for 15–22 million years (Bourguignon et al. 2016). Due to anthropogenic activity, these species have spread globally and established in urban habitats in subtropical and tropical regions (Evans et al. 2013; Gay 1969; Scheffrahn and Su 2005). Both species were reported to be sympatric in Taiwan,

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Hawaii, and Florida (Li et al. 2009; Su et al. 1997; Su et al. 2017; Woodrow et al. 2001), demonstrating their potential for hybridization and viable F1 colony production in laboratory settings (Chouvenc et al. 2015; Lee et al. 2020; Patel et al. 2019a; Patel et al. 2019b; Su et al. 2017).

Although hybridization often directly impacts the potential fitness of hybrid individuals, it may impact eusocial animals at a different level of organization (Fournier and Aron 2021). Social insects such as ants and termites form colonies that display a reproductive division of labor, with the majority of individuals being sterile helpers (workers) (Oster and Wilson 1978). When colonies mature, they produce winged individuals (alates) that disperse and engage in sexual reproduction to establish new colonies (Nutting 1969). Therefore, the potential for hybridization in social insects may only be possible during simultaneous dispersal flight events, for two congeneric species to form heterospecific mating pairs, and potentially establish F1 hybrid colonies.

Termites alates form monogamous mating pairs after dispersal flights (Nalepa and Jones 1991) and males primarily rely on sex pheromones produced by female tergal glands to locate and identify a conspecific partner, to form a tandem and create a new colony (Bordereau and Pasteels 2010). However, this colony foundation process relies on weak sexual selection, as finding a mate and hiding as fast as possible is more important than the potential quality of the partner, owing to strong predation pressure (Chouvenc 2019; Hartke and Baer 2011). The sex pheromones of *C. formosanus* and *C. gestroi* are identical, and their mating behaviors and genital morphologies are also nearly identical (Bignell et al. 2010; Hartke and Baer 2011), potentially leading to accidental heterospecific pairings.

It was suggested that the hybridization between *C. formosanus* and *C. gestroi* may occur in the field from the functional absence of pre-zygotic barriers because, 1) the two termite species have the potential to swarm at the same place and at the same time, 2) the mating choice of both species largely relies on random encounters of alates and 3) both species rely on the same sex pheromones to establish male-female pairings (Chouvenc et al. 2015; Chouvenc et al. 2020; Mizumoto et al. 2020). In addition, hybrid *Coptotermes* colonies from the two possible male-female combinations were established and functional F1 workers were produced in Florida and Taiwan laboratories (Chouvenc et al. 2015; Huang 2020). Termite colonies require five to eight years to reach maturity, posing a challenge in testing the capability of hybrid colonies to produce alates and assessing the fertility of potential hybrid alates. Despite preliminary laboratory studies investigating the biology of termite hybrids, there is currently no evidence indicating the successful establishment of hybrid F1 colonies in the field from heterospecific pairings.

To avoid the emergence of the next superpest, it is important to understand the biology of hybrid populations and to continuously monitor the hybridization dynamics between species (Anderson et al. 2018; Orth et al. 2002; Song et al. 2011; Tay et al. 2013; Valencia-Montoya et al. 2020). Taiwan has the longest sympatric history between the two *Coptotermes* species, among the three known sympatric areas. *C. formosanus* is native to Taiwan (Kistner 1985; Li et al. 2009; Liang et al. 2020; Maruyama and Iwata 2002), while *C. gestroi* invaded Taiwan more than one hundred years ago (Li et al. 2010). It is here hypothesized that if termite hybrid colonies can establish in the field and produce F1 alates, Taiwan would historically be the most likely location to observe it, if investigated.

In this study, we aimed to examine whether (i) the hetero-species colony of *Coptotermes* successfully established in the field and produced alates, and (ii) hybrid alates could establish colony through intercrosses or backcrosses. This study, encompassing extensive field surveys of *Coptotermes* dispersal flight events in Taiwan, utilized molecular identification techniques (Li et al. 2009)

and a citizen science program (Huang et al. 2022). Alates from this study underwent morphological scrutiny based on species-specific criteria (Li 2010; Scheffrahn and Su 2000), with outliers tested for admixed genetic composition from parental *Coptotermes* species. In the 2019–2021 dispersal flight seasons, hybrid alates with atypical morphologies were detected in Taiwan. The investigation extended to exploring alate generations beyond F1 from field dispersal flight events, and the reproductive performance of hybrid alates was assessed in the laboratory.

MATERIALS AND METHODS

Dispersal flight survey and field collection

C. formosanus are distributed island-wide in Taiwan, while *C. gestroi* occupy the Southwestern part of the island (Li et al. 2010). In central-west Taiwan, the ratio of the records of the two species is close to 1:1 (Fig. 1). The city of Taichung was selected as a primary site for dispersal flight surveys because the area has historically displayed a relatively high presence of alates from both *Coptotermes* species when compared to other studied Taiwanese cities (Supplementary Fig. S1). Dispersal flights were monitored and the alates were collected in an area of one square kilometer in Taichung (Supplementary Fig. S2) between April and May of 2019, 2020, and 2021. During the surveys, teams of inspectors canvassed the area with a scooter on a daily schedule. As many dispersing alates as possible were collected by insect nets and were then placed in a container with moist corrugated cardboard. The inspection teams started searching for alates at 18:30 for at least one hour, even in the absence of flying alates. However, if any *Coptotermes* alates were observed in the first hour, the survey was

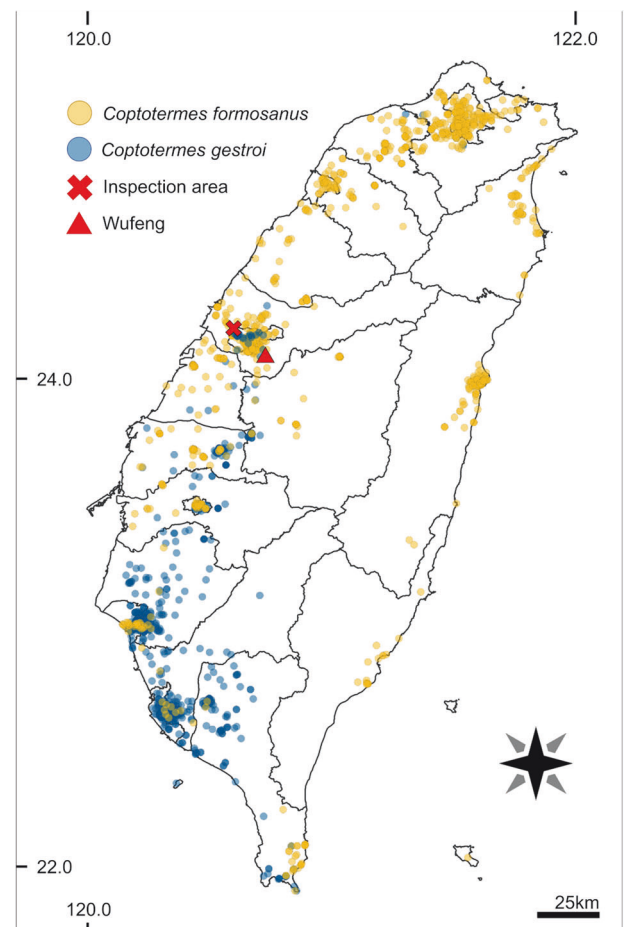


Fig. 1 Distribution of the *Coptotermes* spp. in Taiwan according to NCHU Termite Collection between 2005 to 2020. Locations where natural hybrids were found were denoted as a red triangle and a red cross. Both locations are located in Taichung city, the northern part of the sympatric area.

extended for one additional hour to maximize the sampling effort. We define conspecific individuals collected on the same night within the 1 square km study area as samples of one dispersal flight event. Samples were then brought to the laboratory; voucher specimens were preserved in the National Chung Hsing University (NCHU) Termite Collection with 95% ethanol at -20°C .

In addition to the samples collected from the three-year survey, alates preserved in the NCHU Termite Collection were also examined. In the collection, alates from 682 dispersal flight events were collected between the years 2005 and 2020, partially resulting from a citizen science study (Huang et al. 2022). Alates were classified as either *C. formosanus* or *C. gestroi* when conforming to species descriptions as in Scheffrahn and Su (2000) and Li (2010). The head of *C. gestroi* alates is dark brown, contrasting the two light patches on the face known as antennal spots. In contrast, the head of *C. formosanus* is yellow-brown or orange-brown, and its antennal spots are barely noticeable. Any *Coptotermes* alates that did not fit either of these descriptions were classified as putative hybrids.

Morphological measurements for hybrid identification

Three characters were used to describe the major differences in alate morphology, which were (1) shape of the antennal spots, (2) contrast between the color of the head and the antennal spots, and (3) brightness of the color of the head capsule. For morphological measurements, a total of 115 *C. formosanus* individuals (from 553 samples) and 108 *C. gestroi* individuals (from 129 samples) were randomly selected from the NCHU Termite Collection throughout Taiwan (Supplementary Fig. S3). Additionally, 32 putative hybrid individuals were chosen from samples collected in 2019 from the Taichung inspection area. For detailed sample information, refer to Supplementary Table S1.

Morphological measurements on the antennal spots were conducted using the LAS software (version 4.4.0, Leica Application Suite, Wetzlar, Germany) under a Leica M205 C stereomicroscope (Leica Application Suite, Wetzlar, Germany) mounted with a Leica MC170 HD digital camera. While the antennal spots in *C. formosanus* are oblong, they are crescent shaped in *C. gestroi* and the putative hybrids, convex dorsally and concave ventrally. The vertical height of the crescents (vertical distance between the two vertexes of the borders of the antennal spots) were measured for *C. gestroi* and putative hybrids, and compared by using Student's *t* test ($\alpha = 0.05$).

For color detection and comparison of head capsules, photos were taken in a standard light environment. In LAS software, the output of a light source and the setting of white balance was fixed, exposure 120.0 ms, gain 2.1X, saturation 100.00, and gamma 0.60. The colors of a sample were measured using the software ImageJ (version 1.53e with RGB plugin) and selected ranges for head capsules and antennal spots were measured (8-bit RGB color model, with intensity varying from 0 to 255). The brightness of the color of the head capsule and antennal spots was obtained by summing RGB values, and the contrast between head and antennal spots was quantified through the Euclidean distance between the RGB value of head and spot. Measurements from *C. formosanus*, *C. gestroi*, and the putative hybrids were compared by using the Kruskal–Wallis test followed by Mann–Whitney pairwise test by Bonferroni-corrected *p* values; Past software (version 3.22) (Hammer et al. 2001).

Genetic analysis

The 115 *C. formosanus*, 108 *C. gestroi*, and 32 putative hybrid individuals, all measured morphologically, underwent genetic profiling. Alate DNA extraction modified from HotSHOT method (Meeker et al. 2007) was performed. A set of microsatellite genetic markers established by Chouvenec et al. (2017a) was then used and out of the six microsatellite loci originally assayed, *CopF14F* locus was excluded from the study due to poor PCR amplification, leaving five loci, *CopF6F*, *CopF10F*, *Cg33*, *CopF10-4*, and *Clac1* for subsequent analysis (detailed information of the markers and the PCR conditions see supplementary material and Supplementary Table S2). Loci amplified by PCR were subjected for fluorescent fragment analysis using capillary electrophoresis. Allele calling was performed in the software Geneious (version 2020.2). The allele frequency was scored by using GeneAlec (Peakall and Smouse 2006; Smouse and Peakall 2012).

Microsatellite data was analyzed with STRUCTURE (version 2.3.4, Pritchard et al. 2000). We tested the number of *K* ranging from 1 to 6, and each *K* had 10 replicates with 30,000 burn-in steps and 100,000 data collection steps. Admixture model with correlated allele frequencies was chosen. The optimum *K* was determined using the ΔK method (Evanno

et al. 2005) in a website Structure Harvester (version 0.6.94, Earl and VonHoldt 2012). The threshold *q* value reported in the STRUCTURE analysis was set at 0.1 to identify hybrids (VÅHÅ and Primmer 2006). The program NewHybrids (version 1.1, Anderson and Thompson 2002) was used to assign individuals to 6 classes: pure *C. formosanus*, pure *C. gestroi*, F1 hybrids, F2 hybrids, backcrosses with *C. formosanus*, and backcrosses with *C. gestroi*. Jeffreys-like priors were used, and results were based on the average of 10 independent runs, each with 10,000 burn-in steps and 50,000 iterations.

To detect individuals' maternal lineage, a pair of primers was developed with the mitochondrial genome of *C. gestroi* (Acc# NC_030014) and *C. formosanus* (Acc# NC_015800) and other sequences in Li et al. (2009) as references. The primers flank a *C. gestroi*/*C. formosanus*-specific 11 bp insertion/deletion sequence in the *COII* region (detailed information on the primers and the PCR conditions see supplementary material).

The dispersal season of the hybrid alates

The daily data of dispersal flight events in each of the three years were merged and divided into six time intervals (early April, mid-April, late April, early May, mid-May, and late May). The average number of alates and the average number of flight events were calculated for each time interval.

Because the time-series curves of occurrence observed in previous studies (Chouvenec et al. 2017b; Huang et al. 2022) each contains a single peak with symmetric tails, the curves were estimated by fitting the observed occurrence to a Gaussian function with the nonlinear least square method. The average number of flights at the six periods from April to May was used to estimate the parameters of the Gaussian function. The process of model fitting was performed by using the function *nlsLM* in the R package *minpack.lm*. with the following equation:

$$Occurrence_t = p \times \exp\left(-\frac{(t-T)^2}{2d^2}\right)$$

where *p* is the peak value of the number of flights, *T* is the time at which the peak occurred, and *d* is the deviation controlling the slope of the peak.

Reproductive performance examination

Samples were collected in the dispersal flight survey area in the years 2021 and 2022, live alates were sorted by species and by sex according to morphological traits (Fig. 2), then kept in individual Petri dishes with moistened filter paper. The following colony rearing and dissection protocols were performed within 24 h after collection.

The reproductive performance of different monogamous combinations of the two species and the hybrids were tested. Alates who self-remove their wings (dealate) were paired in each well of the 24-well cell culture plates (Nunclon Delta Surface, Thermo Scientific). In each well, we provided two 1.65 cm diameter fully wetted filter paper (Qualitative Filter Papers No.2, Advantec) as their food and building substrate. After introducing a monogamous pair, a 1.6 cm diameter plastic cylinder (0.47 g in weight) was placed on the top to fill up the cell. The number of laid eggs was determined after eight days of rearing, and the number of offspring (larva, worker, and soldier) was obtained after 54 days. Plates were kept in 100% relative humidity environment, and the average temperature was $29.5 \pm 0.8^{\circ}\text{C}$. The two species and the hybrids were abbreviated as *Coptotermes formosanus*, CF; *Coptotermes gestroi*, CG; hybrid, HY. The pairing combinations were abbreviated as female \times male (e.g. "CG \times HY").

The development of ovaries in each pairing combination was quantified. Female individuals each before and after rearing were randomly selected, and the ovary dissection was performed in PBS buffer (1X, pH 7.4). Ovarioles were carefully loosened by using forceps, and each development level of the ovarioles was determined according to its basal oocyte (Supplementary Fig. S4). The development of basal oocyte was classified into pre-vitellogenic stage (as stage II in (Nozaki and Matsuura 2021; Su et al. 2014) and stage I–stage III in (Costa-Leonardo et al. 2022)) and vitellogenic stage (as stage III in (Nozaki and Matsuura 2021; Su et al. 2014) and stage IV–stage VI in (Costa-Leonardo et al. 2022)). Only as the basal oocyte reaches the vitellogenic stage (in this study we used the length of the basal oocyte >0.13 mm as criterion), the ovariole matures, and it can then successfully produce an egg. Data collected from all the surviving pairings were compared using the Kruskal–Wallis test, followed by Mann–Whitney pairwise tests with Bonferroni-corrected *p* values, using Past software (version 3.22).

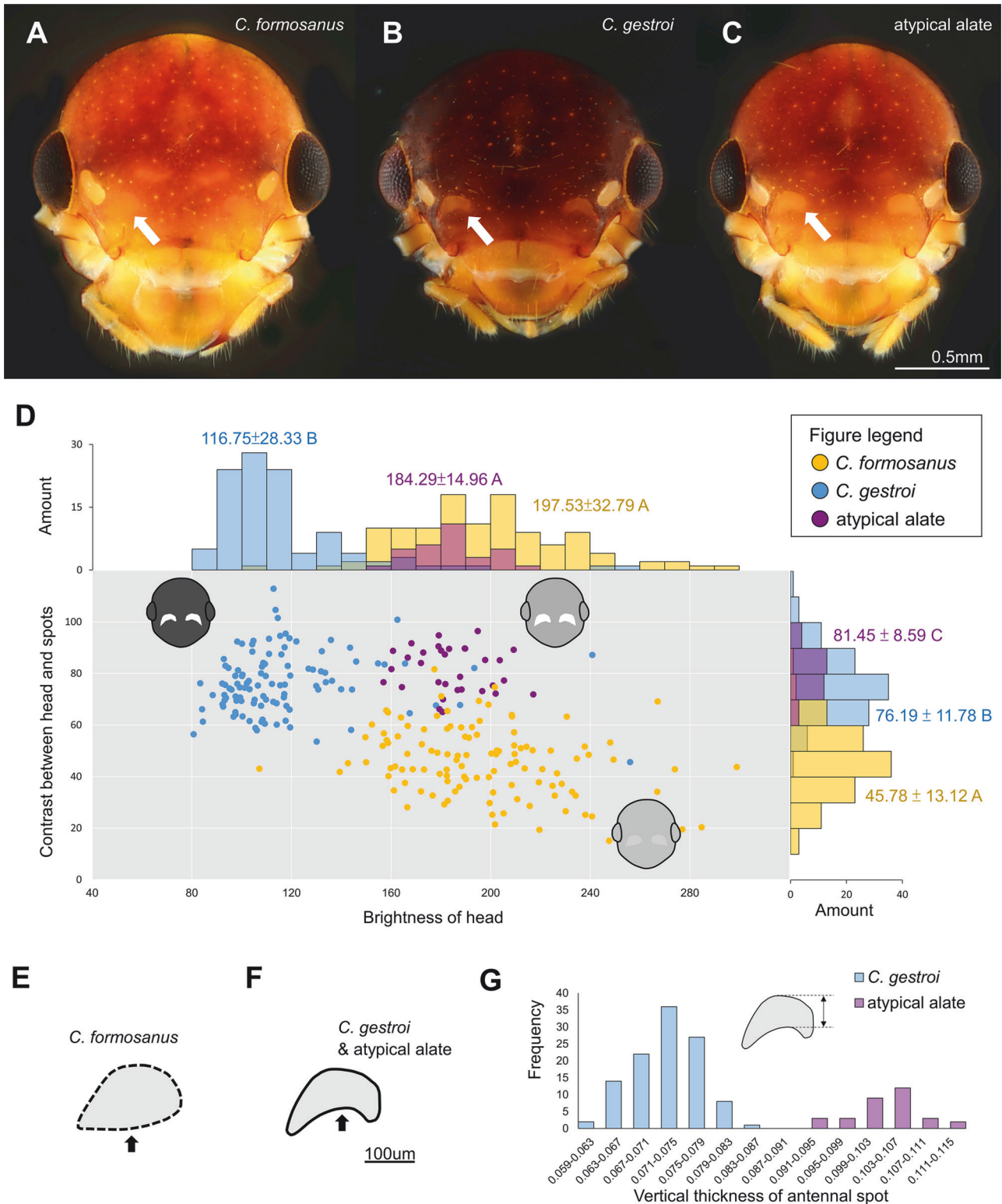


Fig. 2 Facial morphology of *C. formosanus*, *C. gestroi* and the putative hybrids. **A** *C. formosanus*, head saddle brown, no antennal spots or only dim patches. **B** *C. gestroi*, head coconut brown (darker brown) with apparent antennal spots. **C** The putative hybrids, head saddle brown with apparent antennal spots. **D** Morphometric distribution of Taiwanese *Coptotermes* alates. The unit of both axes is the light intensity value measured under the 8-bit RGB color model. The brightness of head (x axes) was obtained by summing RGB values, and the contrast between head and antennal spots (y axes) was quantified through the Euclidean distance between the RGB value of head and spot. The same capital letter after the measurements are not significantly different at the $\alpha = 0.05$ level for each character. **E** The antennal spot of *C. formosanus*, the border of the spot blurry, and if present, both the dorsal and the ventral borders convex. **F** The antennal spot of *C. gestroi* and the putative hybrids, with the dorsal border convex and the ventral border concave. **G** *C. gestroi* and the putative hybrids showed a distinct thickness of the antennal spots.

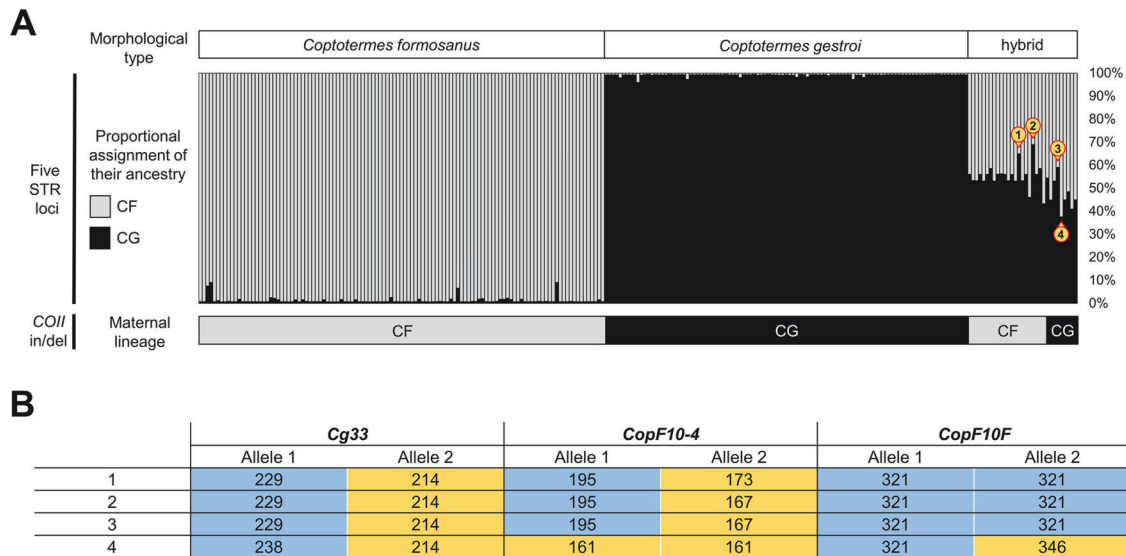


Fig. 3 Species clustering and detection of advanced hybrids. **A** The result of STRUCTURE analysis for $K = 2$ (middle), side by side with individual morphological type (top) and their maternal lineage (below). The result referred to the two morphological types to the two species: the color gray represents the genetic composition from *C. formosanus*, and the color black represents the genetic composition from *C. gestroi*. The putative hybrids carried about half the proportion from each cluster, accompanied by mitochondria inherited from either species, which demonstrates they are the hybrids of the two parental species. The advanced hybrids were marked with number. **B** The alleles of three microsatellite markers of the four advanced hybrids were shown, and the species' private alleles were marked with color. These individuals had alleles on one locus originating from the same species (same color).

RESULTS

Hybrid alate morphology from the alcohol-preserved samples

Three morphotypes were recognized among Taiwanese *Coptotermes*, from the 2019 survey and NCHU collection: *C. formosanus* (Fig. 2A), *C. gestroi* (Fig. 2B), and atypical individuals (Fig. 2C). The morphology of *C. formosanus* and *C. gestroi* samples in this study were both conformed with their respective species description (Li 2010; Scheffrahn and Su 2000). Two facial characteristics, brightness of head (sum of RGB values) and contrast between the head and antennal spots (Euclidean distance between the RGB value of head and antennal spots), were measured in the three morphotypes (Fig. 2D). Alates of *C. formosanus* are light brown in head color (sum of the RGB values, 197.53 ± 32.79), and their antennal spots, if visible, are oblong and dim (Euclidean distance between head and spots, 45.78 ± 13.12), with blurred borders (Fig. 2E). In comparison, alates of *C. gestroi* are dark brown in head color (RGB sum, 116.75 ± 28.33), which contrast drastically with light colored and crescent-shaped antennal spots (Euclidean distance, 76.19 ± 11.78), forming a defined border (Fig. 2F). Atypical alates did not fit either of the above descriptions. They displayed a relatively light head color like *C. formosanus* (RGB sum, 184.29 ± 14.96 , $p = 0.08$) but antennal spots were apparent like *C. gestroi* (Euclidean distance, 81.45 ± 8.59), which were numerically even more apparent than both *C. gestroi* ($p = 0.03$) and *C. formosanus* ($p < 0.001$). In addition, the antennal spots of atypical alates (0.102 ± 0.005 mm) were thicker than those of *C. gestroi* (0.072 ± 0.005 mm, Fig. 2G). Here, we demonstrated the morphological differences of these atypical alates compared to *C. formosanus* and *C. gestroi*; these atypical alates are labeled as "putative hybrid" in the following paragraphs.

In 2019, 2020, and 2021, *Coptotermes* alates were observed for 24, 19, and 13 nights, respectively. Additionally, dispersal flight events for *C. formosanus*, *C. gestroi*, and putative hybrids were observed for 39, 30, and 15 nights, respectively, resulting in the collection of 9829 individuals of *C. formosanus*, 16,556 individuals of *C. gestroi*, and 1218 individuals of putative hybrids. Most of the hybrid alates collected in this study were females, 1:9.9 (male:

female). Of the 682 sampling events preserved in the NCHU Termite Collection, 553 were identified as *C. formosanus*, 128 were identified as *C. gestroi*, and only one was identified as a putative hybrid. The exact number of individuals was not recorded in NCHU Termite Collection.

Genetic analysis

All the five loci, *CopF6F*, *CopF10F*, *Cg33*, *CopF10-4*, and *Clac1* were amplified in *C. formosanus* alates (115/115 individuals, 100%), in *C. gestroi* (103/108, 95.3%), and in the putative hybrids (31/32, 96.8%). Only the individuals which had all loci successfully amplified were used to perform STRUCTURE analysis. In the STRUCTURE analysis the best number of population clusters $K = 2$ (Supplementary Fig. S5). The clustering result indicated an obvious association between morphotype and genotype (Fig. 3). All individuals with *C. formosanus* morphology that carried *C. formosanus* specific mitochondria were assigned to the same cluster (Fig. 3, the proportion of color gray > 0.9); and all individuals in the *C. gestroi* morpho-type carrying *C. gestroi* specific mitochondria were assigned to the second cluster (Fig. 3, the proportion of color black > 0.9). The individuals bearing atypical morphological appearance (the putative hybrids) were assigned as hybrid which displayed admixed composition with q value > 0.3 on both clusters. The evidence that their mitochondrial genotype were from either *C. formosanus* or *C. gestroi* further support their hybrid status from either heterospecific mating combination.

Private alleles, alleles that are found only in a single population among a broader collection of populations, were sorted out (Supplementary Table S2). The hybrid generation was inferred by identifying alleles inherited from *C. formosanus* and *C. gestroi* (Supplementary Fig. S6). For a given locus, the first generation (F1) hybrids are obligately heterozygous for species-specific alleles. If two alleles were specific to the same species, one can rule out the possibility of such an individual as an F1 hybrid. By inspecting all the allele combinations of the 31 hybrids, four individuals were classified as "hybrids of more than the first generation and backcrosses ($> F1$)" (Fig. 3, Supplementary Figs. S6 and S7).

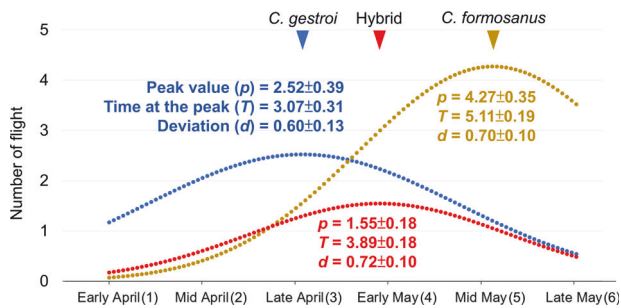


Fig. 4 Time series of dispersal flight events by fitting curves to Gaussian functions. Each curve, *C. formosanus* (yellow), *C. gestroi* (blue), and the hybrid (red), presented one single peak. Arrows point out the timing that the peak value occurred. Gaussian function summarized the curves with three parameters, including p , the peak value of the occurrence; T , the timing that the peak value occurred; and d , the deviation that controls the slope of the peak.

Dispersal flight events and time series curves

The dispersal flight events in the three consecutive monitored years (2019–2021) were recorded, and the average number of dispersal flight events at six defined time periods (early April, mid-April, late April, early May, mid-May, and late May) were counted (Fig. 4A). Dispersal flight models were obtained by fitting the Gaussian function curves (Fig. 4B). For the hybrids and the two parental species, each curve exhibited a single peak and had a shape that suggested a normal distribution (for all parameters, p -values < 0.05). The occurrence of *C. gestroi* reach the peak around late April; followed by the hybrids reaching the peak around early May, and *C. formosanus* reach the peak around mid-May (Fig. 4B). Hybrid alates were frequently observed during simultaneous dispersal flights of either parental species: among the 15 times of hybrid dispersal flight events, 13 times (87%) were concurrent with either or both parental species.

Reproductive performance examination

In the years 2021 and 2022, all possible mating combinations were established as incipient colonies in individual rearing units from live alates collected from field dispersal flight events (details for incipient colony replication for each mating combination were provided in Supplementary Table S3). Before pairing (Fig. 5A), *C. formosanus* females harbored the largest number of matured ovarioles (13.7 ± 1.4), followed by *C. gestroi* females (11.6 ± 1.2 , $p = 0.003$), while hybrid females harbored only one or no matured ovarioles ($p < 0.001$, comparing with both species). After eight days of rearing (Fig. 5B), colonies produced eggs but none had yet hatched. CF \times CF colonies (13.9 ± 1.7) produced significantly more eggs than CG \times CG colonies (8.3 ± 2.1). The F1 colonies developed as well as their maternal species; CF \times CF and CF \times CG had a similar number of eggs ($p = 1$), and so did CG \times CG and CG \times CF ($p = 1$). Backcross between hybrid males and parental species produced a less or equal number of eggs than the conspecific colonies (between CF \times HY and CF \times CF, $p < 0.001$; between CG \times HY and CG \times CG, $p = 1$). Colonies led by hybrid females (HY \times HY, HY \times CF, HY \times CG) developed the worst, they produced less than 1 egg on average (ranging from 0 to 3).

After 54 days of rearing (Fig. 5C), the number of matured ovarioles of hybrid females was still less than 1 on average (ranging from 0 to 4), and was significantly lower ($p < 0.001$) than that of *C. formosanus* females (16.3 ± 1.9) and *C. gestroi* females (14.4 ± 2.5). Eggs hatched and grew into larvae, workers, or soldiers after 54-day rearing. CF \times CF colonies (20.5 ± 8.2) produced significantly more eggs than CG \times CG colonies (13.7 ± 7.0 , $p < 0.001$). The F1 colonies developed as well as their maternal species; CF \times CF and CF \times CG had a similar number of offspring ($p = 1$), and so did CG \times CG and CG \times CF ($p = 1$). Although developing embryos were

observed in the colonies of hybrid male backcrosses (CF \times HY and CG \times HY, Supplementary Fig. S8E, F), only a few CF \times HY colonies (7/22) had their eggs hatched (offspring number ranged from 1 to 22). Finally, none of the eggs produced by hybrid females hatched and no developing embryos were observed after 54 days of rearing (Fig. 5D).

The few eggs laid by hybrid females were malformed and unviable. Compared to the bean-like eggs found in the colonies led by *C. formosanus* and *C. gestroi* females (Supplementary Figs. S9A, B and S8A, D–F), the shape of eggs produced by hybrid females were highly variable. They varied from elongated-bean shape, spherical to short oval, and these eggs were usually smaller in volume than those laid by *C. formosanus* and *C. gestroi* (Supplementary Fig. S9C–U). Eggs laid by *C. formosanus* and *C. gestroi* females contained numerous evenly distributed small yolk granules and lipid droplets. After one week, the developing embryo could directly be observed when laid by a parental species female (Supplementary Fig. S8A, D–F). However, hybrid-female-produced eggs were irregular in internal structure, the yolk or lipid spheres were unevenly distributed and obviously larger than those of parental female species-produced ones (Supplementary Fig. S8B, C).

Summing up the above findings, the colonies led by the females of either parental species grew rapidly with the emergence of workers and soldiers, both in conspecific (CF \times CF, CG \times CG) and heterospecific (CF \times CG, CG \times CF) mating combinations. In comparison, only 7 out of a total of 22 colonies, where hybrid males were backcrossed with *C. formosanus* females, could develop and produce offspring; meanwhile, all five colonies where hybrid males were backcrossed with *C. gestroi* females failed. Hybrid females displayed abnormal ovariole morphology and produced oocytes that seldom developed further than stage II. Even if the eggs were laid, their internal structure was irregular, leading to embryos' nonviability.

DISCUSSION

Field-established F1 hybrid *Coptotermes* colonies can produce alates

This study revealed that the dispersal flight seasons of *C. formosanus* and *C. gestroi* overlapped within a 3-months period in Taichung, Taiwan, during the three consecutive years of field observations from 2019 to 2021. It emphasizes an ongoing opportunity for both species to engage in heterospecific mating behavior in the field (Chouvenc et al. 2020), as previously observed in Florida (Chouvenc et al. 2017b). The potential for hybridization (F1) between the two *Coptotermes* species was only previously confirmed in a Florida laboratory from the successful rearing of heterospecific colonies (Chouvenc et al. 2015). Such hybrid colonies may grow at least equally fast to either of the parental species, but it was noted that F1 hybrid workers could display altered molting rates (Lee et al. 2020), which raised questions on the abilities for F1 hybrid colonies to ever reach maturity and produce viable F1 alates. However, the current study provides the first evidence of *Coptotermes* hybrid alates during dispersal flights, therefore indirectly confirming the existence of field-established F1 *Coptotermes* hybrid colonies in Taichung.

During the dispersal season of hybrid alates, there was an overlap with the two parental *Coptotermes* species, and the peak occurred in early May, between *C. gestroi* (late April) and *C. formosanus* (mid-May) (Fig. 4). Over a three-year alate sampling experiment, out of 27,601 alates collected, 1218 (4.4%) were confirmed hybrid alates. In most cases, hybrid alates were simultaneously collected with alates of either parental species (13/15 times). Therefore, while hybrid alates may engage in mating behavior among each other, our observation also opens the possibility for hybrid alates to backcross with alates from either parental species. Anecdotally, most hybrid alates collected

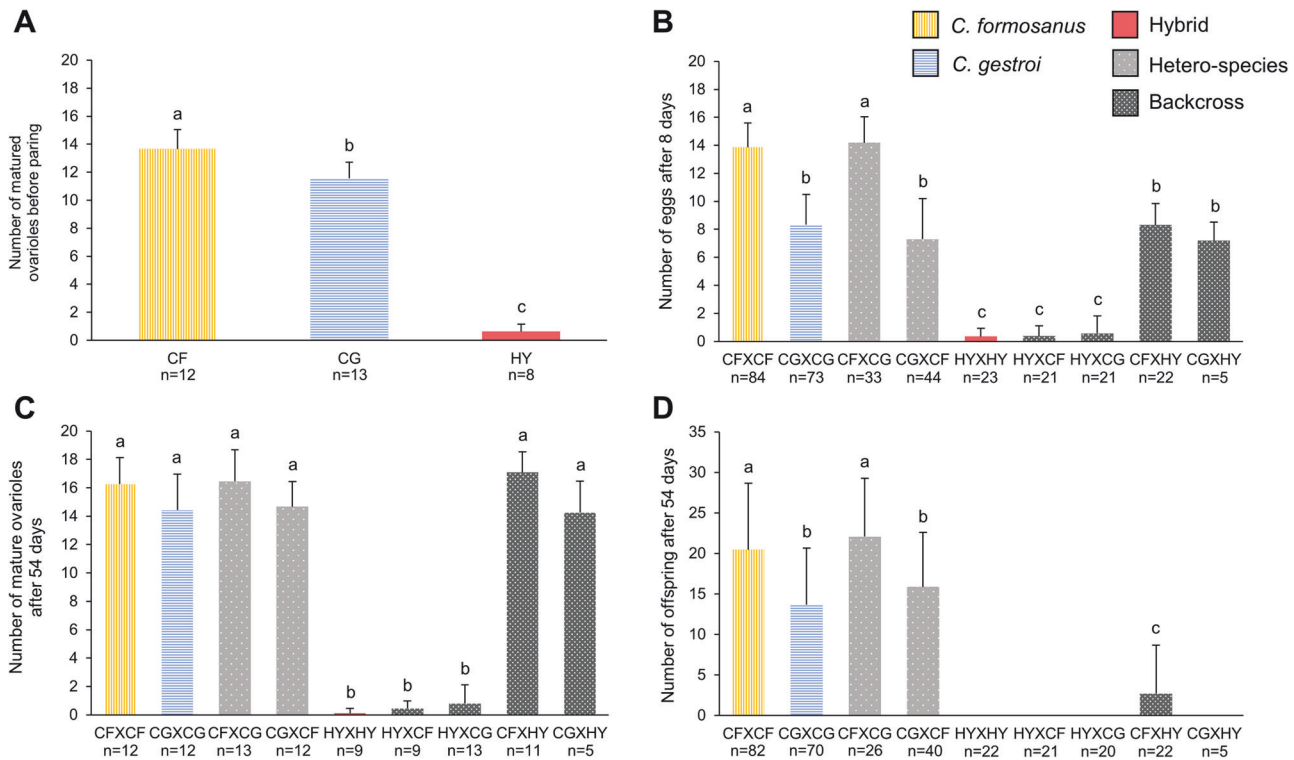


Fig. 5 Reproductive performance of the incipient colonies. **A** The average number of matured ovariole before pairing. **B** The average number of eggs after eight days of rearing. **C** The average number of matured ovariole after 54 days of rearing. **D** The average number of offspring after 54 days of rearing. Comparisons were done using the Kruskal–Wallis test followed by Mann–Whitney pairwise test by Bonferroni-corrected p values. When columns are marked as the same letter, the means are not significantly different at the $\alpha = 0.05$. The pairing combinations were abbreviated as female \times male. CF *C. formosanus*, CG *C. gestroi*, HY hybrid.

in this study were females, 1:9.9 (male: female), $n = 1218$ suggesting three possible scenarios: 1) sex ratios of all laid eggs may be altered in F1 colonies, 2) F1 male alates may have poor viability compared to female alates, or 3) F1 male alates may be relatively poor fliers, preventing them from being collected during dispersal flights. Regardless of the cause of the observed hybrid alate sex ratio which remains to be further investigated, this preliminary observation would imply that backcross mating opportunities beyond F1 may primarily be possible through female hybrids. However, incipient colony growth results from all potential mating combinations suggest otherwise, as discussed below.

As observed in laboratory rearing using Florida *C. gestroi* and *C. formosanus* populations (Chouvenc et al. 2015), we confirmed that the Taiwanese *Coptotermes* populations also have the ability to produce viable hybrid colonies in laboratory conditions. Queens in incipient colonies of both conspecific and heterospecific mating combinations were able to lay viable eggs, with the royal pair rearing their first offspring through biparental care and ultimately switching to alloparental care for further colony growth (Chouvenc 2019; Chouvenc 2022). We further conducted a backcross experiment to test the ability of hybrid alates collected from the field to establish incipient colonies when paired with either parental species in the laboratory. While all hybrid females displayed extremely reduced egg-laying abilities and none of their laid eggs were viable, some hybrid males within CF \times HY pairings (7/22) were able to produce viable offspring and successfully establish as incipient colonies. Therefore, our results confirmed an overall relatively poor fertility in hybrid alates, with the inability of hybrid females to produce viable eggs, but with partial fertility in hybrid males. This observed asymmetrical viability and fertility may have important consequences in the potential gene flow between the two species, as on the one hand,

the hybrid alate sex ratio is strongly biased toward hybrid females, but on the other hand, only small proportion of hybrid males may have the possibility for backcrossing. Thus, these two compounding observations would suggest that the probabilities for advanced-hybrid colonies to be established in the field may critically be reduced.

Four individual hybrid alates collected from the field revealed combinations of homospecific and heterospecific alleles (combination of private alleles from the same species or not), demonstrating the existence of F2 or backcrossed colonies in the field (Fig. 3, Supplementary Figs. S6 and S7). We here hypothesize that during simultaneous dispersal flight events involving F1 alates, most of them fail to establish viable colonies when compared to alates from parental species. However, through a number's game of genetic combinations, a limited number of attempts may eventually succeed, allowing for further genetic introgression.

Male *C. gestroi* preferably engage in mating behavior with *C. formosanus* females, while *C. formosanus* males primarily ignore *C. gestroi* females (Chouvenc et al. 2020). It was therefore assumed that if established in the field, hybrid colonies would inherently carry a *C. formosanus* maternal lineage and that genetic introgression would be asymmetrical within the two parental species (*C. gestroi* genes flowing into *C. formosanus* populations, but not the other way around) (Chouvenc et al. 2015; Chouvenc et al. 2020). However, our results disproved this assumption, as hybrid alates with either maternal lineage were found in the field (Fig. 3), confirming that female *C. gestroi* \times male *C. formosanus* mating events also occurred in the field. It therefore maintains the possibility for a symmetrical genetic introgression between the two parental species, although such probabilities remain relatively low owing to the apparently poor F1 hybrid alate fertility.

Hybrid identification

Scheffrahn and Su (2000) provided criteria for morphological identification of the alates of the two *Coptotermes* species, which have been in use for over two decades. In the current study, we have provided a quantitative basis for morphological characterization of alates of the two *Coptotermes* species and also for their hybrid. In 95% ethanol-conserved individuals, the brightness of the head capsule, its contrast with antennal spots, and the shape of the antennal spots were three valid characteristics to morphologically identify each of them. When individuals were collected alive (not dried out or preserved) and placed side-by-side, a visible color difference on the abdomen was detected with the naked eye (Supplementary Fig. S10). Dorsally, the abdomen of hybrid alates is nutty brown, in *C. formosanus* is orange (lighter), and in *C. gestroi* is dark leather brown (darker). Hybrid alate abdomens are therefore intermediate in hue between the two parental species, but because of inherent color variability after death, desiccation, or ethanol preservation, this characteristic can only be reliable when live individuals of the three types are available for comparison. In cases where morphological characteristics for identifying alate samples are absent or if the sample has clearly undergone a color change, hybrid specimen identification can also be confirmed through the molecular approach described herein.

Although our research establishes a strong correlation between morphology and genetic composition, it is crucial to bear in mind that advanced hybrids and backcrosses may not consistently exhibit intermediate phenotypes or adhere to a linear genotype-phenotype relationship (Rieseberg et al. 2003; Satokangas et al. 2023). Therefore we remain open to the possibility that some hybrid individuals could not be identified by morphological evidence and that certain advanced hybrids and backcrosses might not have been detected due to the limited number of the markers we used.

Hybridization, past and future

The identical sequences of COII, 12S rRNA, and 16S rRNA sequences in *C. gestroi* population in Taiwan indicates that the species underwent a recent invasion event (Li et al. 2009). Historical records suggest that the termite species entered Taiwan over a century ago via ancient ports in Southern Taiwan. (Andrade 2008; Li et al. 2009; Scheffrahn and Su 2005). Nowadays, *C. gestroi* is the most commonly reported termite species in Southern Taiwan, comprising over 90% of citizen-reported cases (Huang et al. 2022). This suggests that the species has rapidly expanded and outcompeted the native species *C. formosanus* in the southern part of the island (Fig. 1). During the expansion period, the two species should have mated, resulting in admixed populations. However, among the 682 samples gathered by citizen scientists from all over Taiwan Island (NCHU Termite Collection), only one hybrid sample was identified in central Taiwan, the remaining samples were identified as either *C. formosanus* or *C. gestroi* (Fig. 1). The absence of hybrid population in the south, where the two species have coexisted for the longest time, supports the hypothesis that the hybrids were unfit. This suggests the possibility that either all admixed populations that once existed died out due to hybrid breakdown, or the genetic components of *C. formosanus* were progressively eliminated from the genomes of the admixed populations, leaving behind the genomes of *C. gestroi* (Moran et al. 2021).

From an evolutionary perspective, hybridization may lead to three primary outcomes. (1) Hybrid speciation: hybrid lineages become reproductively isolated from the parental species for various reasons, resulting in a new population with mixed ancestry derived from both parental populations (Mallet 2007). (2) Fusion: a breakdown of species barrier occurs as the two distinct gene pools of hybridizing species homogenize, leading to a 'reverse speciation' within a geographical 'hybrid zone' (Maan et al. 2010; Taylor

et al. 2006). (3) Reinforcement: due to poor hybrid vigor and fertility, prezygotic barriers may strengthen, reducing the likelihood of forming unfit hybrids (Liou and Price 1994; Servedio and Noor 2003). In the current study we documented severe postzygotic barriers in hybrids of *C. formosanus* and *C. gestroi*. Considering both the severe postzygotic barriers and the absence of a hybrid population in southern Taiwan, we propose that the reinforcement scenario should apply to *Coptotermes* populations in all sympatric areas.

In the context of hybridization in mice (*M. m. domesticus* × *M. spretus*) and moths (*H. zea* × *H. armigera*), only a fraction of female F1 hybrids in mice are fertile (Dejager et al. 2009), with a mere 1–2% chance of F1 hybridization success in moths (Laster and Sheng 1995). Nevertheless, genetic introgression has occurred in the natural populations (Song et al. 2011; Valencia-Montoya et al. 2020). Despite apparently poor fertility of F1 hybrid alates, the potential for genetic exchange between the two termite species persists. The temperature tolerance ranges for *C. formosanus* and *C. gestroi* were 15–30 °C and 20–35 °C, respectively (Cao and Su 2016; Patel et al. 2019). Previous studies have demonstrated that hybrid termites produced in the lab can survive in the combined ranges of both parental species, 15–35 °C. (Patel et al. 2019), indicating that hybridization can serve as a basis for adaptation to a distinct temperature range. Additionally, the foraging strategies of the two species differ in terms of tunneling patterns and food preferences (Hapukotuwa and Grace 2011; Hapukotuwa and Grace 2012). Hybridization among these species may not only impact their temperature tolerance but also foster the development of unique foraging strategies, ultimately contributing to the expansion of their adaptability and invasiveness.

The presence of more than one generation of hybrid *Coptotermes* alates in the field suggests the potential gene flow in regions where both termite species coexist. This discovery underscores the necessity for expanded survey efforts, not only in southern parts of Taiwan but also in regions such as Florida and Hawaii, to detect hybrid *Coptotermes* alates. Comprehensive studies should also be undertaken in all sympatric areas to conclusively determine the extent of possible genetic introgression resulting from hybridization and backcrossing. Finally, this study reveals that hybrid *Coptotermes* can serve as a relevant model system to study the effect of hybridization in a diploid eusocial insect.

DATA AVAILABILITY

Supplementary figures, tables, and text can be downloaded from the online article version. Input files for population genetic analyses can be found on Dryad (<https://doi.org/10.5061/dryad.n2z34tn4g>).

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AUTHOR CONTRIBUTIONS

GYC, SYH, YHC and HFL designed the study. GYC and SYH collected the data. GYC, YHC, MDL and HFL interpreted the results. GYC analyzed the data and wrote the manuscript. YHC, MDL, TC and HFL revised the manuscript. HFL acquired funding for the project. All authors read and approved the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS

The research involved insects only and was exempt from ethical approval.

ADDITIONAL INFORMATION

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