**Loss of pair formation predates the evolution of male-less society in *Glyptotermes* termites**

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**Abstract**

Parthenogenesis and the loss of males have occurred repeatedly across diverse organisms. Asexually reproducing lineages are not usually associated with social animals that exhibit biparental care because such care is inherently linked to the behavioral sequence of mate pairing and sexual reproduction. The male-less lineages of the termite, *Glyptotermes nakajimai*, provide a rare opportunity to study how sexual reproduction can be lost in social animals with parental care. Here we demonstrate that modification of the mate-pairing process predated the evolution of asexual lineages. Termite colonies are typically founded by a mating pair, with many species forming a tandem courtship pair while searching for a nest site. Our comparative analysis of tandem running in *Glyptotermes* termites revealed that two related species, *G. fuscus* and *G. satsumensis*, exhibited both female-leader and male-leader tandem runs, estimated to be the ancestral state in this genus. On the other hand, tandem running was rare and ephemeral in both sexual and asexual lineages of *G. nakajimai*. These results suggest that *G. nakajimai* employs an alternative colony foundation strategy, as further supported by their colony structures. Our study highlights the tight link between the evolution of asexuality and behavioral preadaptation, contributing to our understanding of the evolution of complex phenotypes.

**Keywords:** Asexual reproduction, Movement coordination, Parental care, Same-sex sexual behavior, Social insects

**Introduction**

Sexual reproduction is the norm for multicellular organisms despite the costs of producing males [1,2]. Many studies have revealed the advantages of sexual reproduction in short and long periods (e.g., [3]). Furthermore, in established sexual species, there are genetic constraints to prevent the evolution of asexuality by maintaining a low transition rate from sexual to asexual reproduction [4]. Therefore, although asexual lineages evolved across the diversity of taxa, the evolution of the asexual population is relatively rare. In addition to genetic mechanisms, specific behaviors of sexual organisms can prevent the evolution of asexual lineages, such as sexual conflicts [5] (but see [6]). Among behavioral traits, the evolution of asexuality is challenging in social animals with parental care because sexual reproduction is associated with the sequence of mate pairing and biparental care, where males contribute to fitness more than sperm [1,7].

Termites evolved from the ancestor of subsocial cockroaches, and colonies also started from subsocial pairs [8,9]. During a season of the year, alates (winged reproductives) fly to disperse to find a mating partner [10]. After shedding wings, many termite species form tandem pairing; a female and a male walk together while searching for a nest site [11]. In colony foundation processes, males significantly contribute to the offspring energetically and through physical labor [12,13]. Also, termites require a partner for allogrooming, making nest establishment as a single termite unsuccessful [14,15]. Therefore, even if several termites have parthenogenetic ability [16–18], they need a female partner for colony foundation. A species of drywood termite, *Glyptotermes nakajimai* Morimoto (Isoptera: Kalotermitidae) [19,20], provides a unique opportunity to study the evolution of asexual lineages in animals with biparental care. In several populations, this species completely lost males, and all colonies are comprised only of females (i.e., all-female asexual societies) [21]. The evolution of this population requires the modification of a typical monogamous colony foundation in termites.

Two different potential behavioral preadaptations enable the evolution of a male-less colony foundation in termites. First, colony establishment by female-female pairs after same-sex tandem runs. For example, in *Reticulitermes* termites, same-sex tandem runs function as heterosexual tandem runs [11,22], and female-female pairs start their nests with parthenogenesis [15,23], although they are not very successful in the long term [24–26]. If the ancestor of *G. nakajimai* has strong tandem running behavior with same-sex pairing, they can achieve the evolution of an asexual population without substantially changing mate pairing behaviors. Second, pleometrosis (colony foundation by multiple kings and queens) can also facilitate the evolution of an asexual society by securing the number of colony foundation partners [27]. Actually, the colonies of *G. nakajimai* are often headed by multiple reproductive [21]. However, how they achieve such pleometrosis remains unknown; do they use ancestral tandem running to form multiple pairs or completely modify the pairing process as preadaptation?

In this study, we investigated the mate-pairing process of three different *Glyptotermes* species (*G. nakajimai, G. fuscus, G. satsumensis*) by confirming their phylogenetic relationship. These three species can be found sympatrically and relatively related to each other [19,28], but asexual society only arose in *G. nakajimai*. We especially focused on tandem running behavior by automatically quantifying it using the deep-leaning automated posture-tracking software [29]. We also estimated their phylogenetic relationship using mitochondrial genomes. By mapping their ecological and colony structure information on the phylogeny, we reconstruct the behavioral aspects of the evolution of a male-less society.

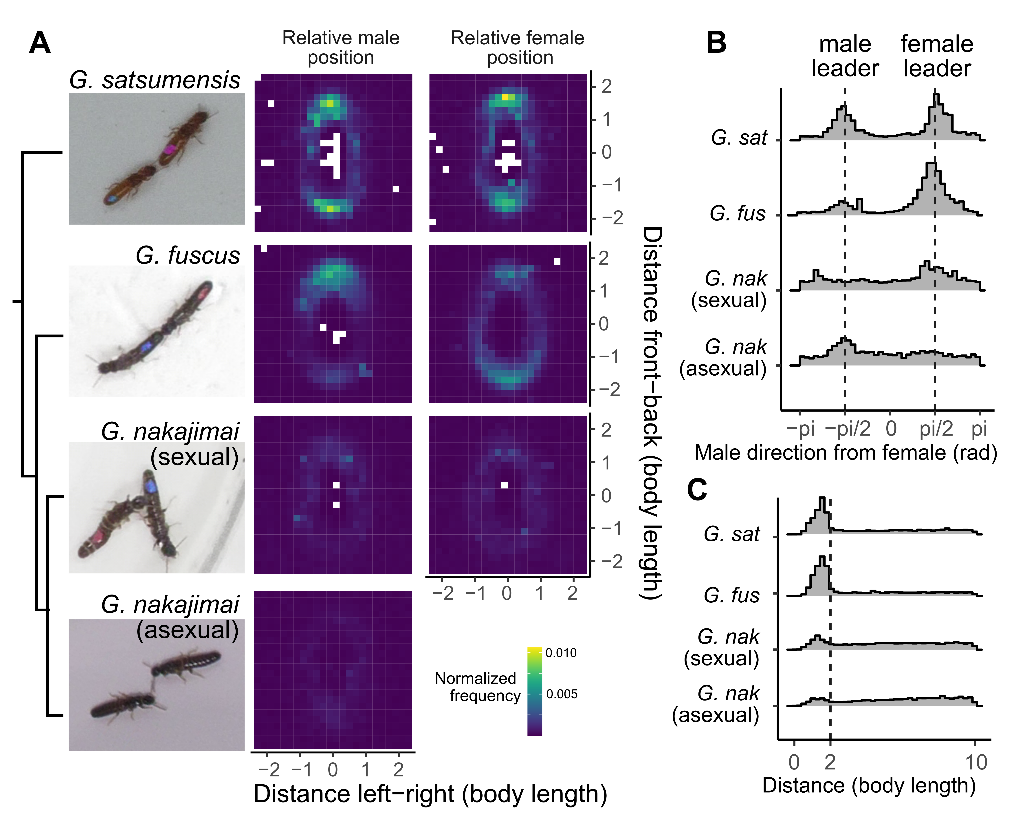
**Results and Discussion**

*Observation of tandem pairing behavior*

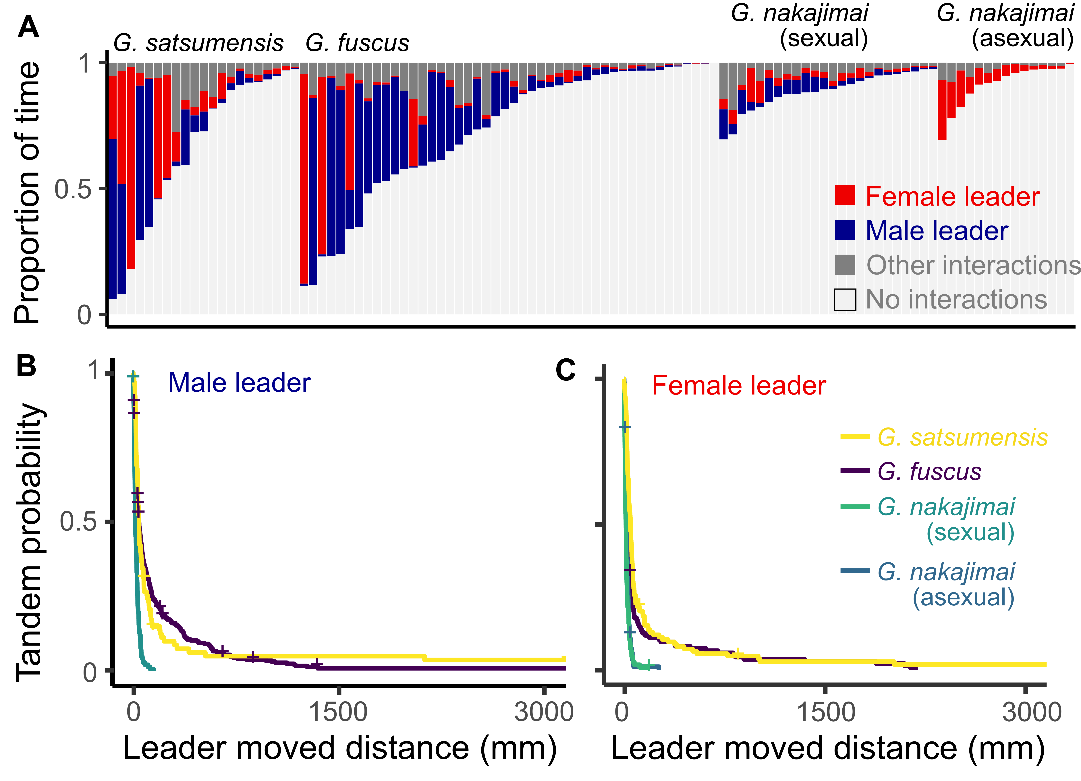
Tandem running behavior was clearly observed in *G. fuscus* and *G. satsumensis*, while it was unclear in *G. nakajimai* from both sexual and asexual populations (Figs. 1 and 2). First, these species showed distinct patterns in pair positioning when we observed behavioral interactions of mating pairs (or female-female pairs in asexual populations) in a pest dish arena. When we pooled the datasets within each species, pairs were found often within two body length distances across all species, where data distribution seemed to follow different patterns within or beyond two body lengths (Fig. 1AC). Thus, termites should have been in interaction when they were within two body lengths. Second, when termites were within two body lengths, spatial orientations of pairs were different. In *G. satsumensis*, pairs were often positioned in a single file, where females were either behind or in front of the males, and vice versa (Fig. 1AB). In *G. fuscus*, similarly positioned in a single file; females were often positioned behind males (Fig. 1AC). In contrast, we could not observe the clear following positioning in *G. nakajimai* in either sexual or asexual populations (Fig. 1AB). We used these observations for defining tandem runs; partners were in interaction when the distance was within two body lengths, and during interactions, pairs were in tandem runs when the female was in front of the male and the male was behind the female (and vice versa).

Next, we looked into the details of the pairwise interactions for each pair. Tandem running behavior was highly variable across different pairs, even with the same species (Fig. 2A); some showed tandem runs during entire observations, while some did not show tandem runs at all. However, long tandem running (e.g., more than 40% of the observational time) was only observed in *G. satsumensis* (7/21) and *G. fuscus* (8/46), not in *G. nakajimai*. There was no significant difference in the proportion of the time for tandem running behavior among species (GLMM, χ23 = 3.74, *P* = 0.291). Within each species, there were differences in the proportion of time spent in tandem runs, where the female-led tandem run was more common than male-led in *G. satsumensis* (GLMM, χ21 = 22.92, *P* < 0.001), while male-led tandem runnings were more common than female-led tandem runnings in *G. fuscus* (χ21 = 26118, *P* < 0.001) or *G. nakajimai* (χ21 = 497.38, *P* < 0.001). Thus, the male is the more active sex for pairing in *G. satsumensis*, while the female is the more active sex for pairing in *G. fuscus* and *G. nakajimai*.

Because the function of tandem running behavior is to maintain pair cohesion while exploring the environments, tandem running behavior should be evaluated as the distance a pair moved during a continuous interacting event rather than the duration they spent in the posture of following the other. When we compared the traveled distance, we found that *G. satsumensis* and *G. fuscus* traveled a longer distance than *G. nakajimai* (Fig. 2; Cox mixed effect model, male leader, χ23 = 30.19, *P* < 0.001, female leader, χ23 = 17.56, *P* < 0.001). In this sense, although *G. nakajimai* showed ephemeral tandem runs, their functionality is questioned; even the longest tandem run broke up after moving 185 mm (sexual) and 260 mm (asexual). Given that these species start their nest at the tree branches, they cannot explore among options during pair maintenance. On the other hand, tandem running of *G. satsumensis* and *G. fuscus* can travel for more than 1 m long in 24 events continuously (15: *G. fuscus*, 9: *G. satsumensis*, from 16 pairs, 11: *G. fuscus*, 5: *G. satsumensis*). This allows them to explore the tree branches thoroughly. Within these species, there were no significant functional differences between female-led and male-led tandem runs (Cox mixed effect model, *G. fuscus*; χ21 = 2.41, *P* = 0.121, *G. satsumensis*; χ21 = 0.14, *P* = 0.704).



**Figure 1.** Spatial positioning between partners in *Glyptotermes* termites. (A) Comparison of the relative position of the partner, given that female (left) or male (right) heading towards the top at the center. Simplified phylogenetic relationship based on [21] is also provided. (B) Distributions of the partner's position relative to the female's heading direction in angles when the pair is within 2 body lengths. (C) Distributions of the distance between partners.



**Figure 2.** Tandem running behavior of each species. (A) Proportion of time in each state during observation. Each bar represents one pair. (B-C) Interspecific comparison of the traveled distance during each tandem running event.

*Ancestral state of tandem running behavior*

Previous studies on genus level ancestral state reconstruction and fossil record suggested that the ancestor of Kalotermitidae exhibited tandem running behavior [11,30]. At the same time, tandem running is highly variable within Kalotermitidae, where some showed tandem running, but some do not even within the same genus [11]. To reconstruct the evolutionary process of tandem running, we performed the species level ancestral state reconstruction, using the mitochondrial genome phylogeny and published datasets.

We estimated that the ancestor of Glyptotermes tandem running as follows:

Both leader -> active role of female in pairing -> loss of tandem running ->loss of males

Sexually active males prevent the evolution of asexuality

*Colony structure*

In *G. nakajimai*, colonies often include many alate-derived reproductives in both sexual and asexual populations [21]. Also, in asexual populations, colony foundation by multiple queens has been observed, while no data is available in sexual populations [21]. Such pleometrosis (colony foundation by reproductives more than a pair) should have contributed to removing a barrier to the evolution of asexuality as they can achieve alloparental care without males. Among termite species, pleometrosis has been observed in several species of Termitidae [31–33]. However, in Kalotermitidae, colonies are commonly headed by a monogamous reproductive pair (e.g., [34,35]). Thus, we checked the colony structure of *G. satsumensis* and *G. fuscus* to further investigate the origin of pleometrosis as a preadaptation of asexuality.

Although our records are limited, mature colonies of *G. fuscus* and *G. satsumensis* often included multiple reproductives (Table 1), with sexual populations of *G. nakajimai* having more reproductives than asexual populations or *G. fuscus* (GLM, χ22 = 140, *P* < 0.001, we did not include *G. satsumensis* due to limited sample size). However, the source of such multiple reproductives in mature colonies could have differed from *G. nakajimai* and the other two species. A previous study showed that incipient colonies of *G. nakajimai* already had multiple queens [21] (pair: 1/4), while incipient colonies of *G. fuscus* or *G. satsumensis* were monogamous pairs. (pair: 4/4, Table 1). If they start a colony from a monogamous pair, colony fusion could be a mechanism of multiple reproductives in mature nests, although colony fusion often causes competition between reproductives and results in monogamous pairs in many other species (e.g., [14,36,37]). In this sense, *Glyptotermes* termites might be tolerant to the presence of other reproductive, which may have played a role in facilitating the evolution of pleometrosis in this group.

We detected the correlation between pair formation behavior and colony foundation unit in the studied species; *G. fuscus* and *G. satsumensis* exhibited tandem running and monogamous colony foundation (Fig. 2, Table 1), while *G. nakajimai* lacked them (Fig. 2). However, it remains unclear if tandem pair formation necessarily leads to monogamous pairing. For example, although some other Kalotermitidae species (e.g., *Neotermes* and *Pterotermes*) lack tandem running behavior [38,39], these showed more strict monogamous pairing [34,35]. On the other hand, *Odontotermes* or *Macrotermes* termites show tandem running (summarized in [11]), but they show pleometrosis relatively frequently [31]. Instead, resource availability should facilitate pleometrosis [27]. When the nesting site is a limited and valuable resource, termite dealates must concentrate and be forced to start a colony with many individuals. Actually, the distribution of *G. nakajimai* is highly localized within a specific area compared to the other two species. Pleometrosis observed in *G. nakajimai* may have been due to their particular requirements as a site of colony foundation,

***Conclusion***

Parthenogenesis ability has evolved independently many times in termites [17,18,40], but asexual society only happens in *G. nakajimai* as far as we know. We attributed this rarity to the complex combination of required preadaptations, including females being active sex (Fig. 1), lack of tandem running (Fig. 2), and pleometrosis. For example, *Reticulitermes* termites and several other termites incorporate facultative parthenogenesis into their colony reproductive system and life history [40,41]. Also, these species show female-female pairing and colony foundation [42], although female-female pairs do not result in functional colonies in a long timeline [24,25]. These species ultimately use strict social monogamy, where the presence of multiple kings or queens leads to competition and a monogamous pair. Also, male is the active sex of tandem pairing, which might hinder the evolution of asexuality. Similarly, *Neotermes sugioi* shows facultative parthenogenesis and even lacks tandem running behavior [38,43]. However, this species also shows strict monogamous pairing.

Sexual reproduction is the norm in animal kingdoms, and the evolution of asexuality does not happen randomly across lineages because each group has a specific unique hurdle to overcome during male loss [2,4]. Many theoretical studies of the evolution of sexuality have acknowledged that parental care by males can minimize the cost of sexual reproduction. Thus, evolutionary change of behavior should accompany such species. In this study, we showed that mate pairing behavior went through several steps of preadaptations in the course of the evolutionary loss of males in a termite, *G. nakajimai*. This highlights the importance of behavioral preadaptation as a prerequisite for the evolution of sexuality in social animals. By focusing on the behavioral aspects of sexual and asexual evolution, our results shed light on the integrative approach to the evolution of the sex.

**Methods**

*Termite collection*

We collected all termite colonies with a piece of nesting wood from the field. We collected three colonies of *G. fuscus* (one in January 2021 and one in March 2022 in Nago, Okinawa; one in March 2023, Iriomote Is., Okinawa), three colonies of *G. satsumensis* in March 2021 (two in Minamiosumi, Kagoshima, one in Kushima, Miyazaki), and four colonies of *G. nakajimai* (two in March 2021, Wakasa, Fukui, one in April 2023, Tokunoshima Is. Kagoshima, one in March 2021 in Cape Toi, Miyazaki). For *G. nakajimai*, samples from Fukui and Tokunoshima Is. were sexual [44], while the sample from Cape Toi was asexual [21]. The field collection was performed before the swarming season; each colony contained nymphs but not alates. All colonies were maintained within the nesting wood at 22°C until the experiments. Before each experiment, we transferred nests to a room at 27 °C, which promoted alates to emerge and fly. We also opened the nest with axes and collected alates that flew out of the nest. We induced alates to shed their wings by manually pinching their wings with forceps. Then, we maintained them in Petri dishes containing moist, unwoven cloth in the light and used for the experiments within 1 day of flight. Dealates were separated by sex and color-marked with one dot of paint (PX-20; Mitsubishi) on the abdomen to distinguish sex identities.

*Behavioral observations*

We introduced a female-male pair of termite dealates (female-female pair for an asexual population of *G. nakajimai*) to the experimental arena, consisting of a petri dish (φ = 90 mm) covered with a layer of moistened plaster. All pairs were prepared using nest mates. The effect of intracolonial and intercolonial pairing on tandem running behavior is not known for *Glyptotermes* spp, but we only used nest mate pairing consistently for performing across-species comparison. Note that tandem running is not different between nestmate and non-nestmate pairing in another species [11]. We recorded their behavior for up to 60 minutes at 30 frames per second. All the videos were cropped to 1200x1200 pix to include only the arena in the frame before the video analysis. In total, we observed 21 pairs of *G. satsumensis* (340:16, 347:2, JP21-06:3), 46 pairs of *G. fuscus* (21A:16, G05:18, NM2325:12), 25 pairs of *G. nakajimai* sexual populations (356:6, 367:3, NM2344:16), and 15 pairs of asexual populations.

All videos were analyzed using SLEAP v 1.4.0 [29] to estimate the movement of body parts of each individual. We used a 6-node skeleton: antenna tips (LR), head (middle of mouth parts), head-pronotum boundary, body center, abdomen-tip, and a dot of a color-painted marker. We built a model for one species and then used it as a starting point to build another for the next species sequentially. First, we labeled 342 individuals from 23 videos for training in *G. satsumensis*. We trained a U-Net-based model with a multi-animal top-down approach, with a receptive field size of 76 pixels for the centroid and 156 pixels for the centered instance, on Nvidia GeForce RTX 4090, where augmentation is done by rotating images from -180 to 180 degrees. The mean Average Precision (mAP) and mean Average Recall (mAR) of this model were 0.36 and 0.49, respectively. While tracking after the inference, we used the instance similarity method with the greedy matching method. All pose estimation data were converted to HDF5 files for further analysis. Then, we used the model for *G. satsumensis* as a starting point and developed a model for *G. fuscus* with 956 individuals from 37 videos. This model was further fine-tuned by only using labels for each colony. Similarly, the models of *G. nakajimai* were trained based on this *G. fuscus* model for each colony. All these models are available at XXX.

We used Python to format all HDF5 files for further analysis and converted them into FEATHER files for analysis in R [45]. We employed a linear interpolation method to address missing values in the dataset. After scaling all data from pixels to mm (1200 pixels = arena size), we used a median filter with a kernel size of 5 to reduce noise.

To compare tandem running behaviors among species, we automatically determined whether pairs were in tandem based on the postures and spatial position of partners. First, we regarded as two individuals being in interaction when the distance between the body centers of partners was less than two body lengths, based on the frequency distribution of this distance (Fig. 1C). In this process, we ignored the short interaction events or non-interaction events less than 2 seconds to smooth the data. Second, during interactions, we classified termite heading orientation as female-leader and male-leader. We obtained the heading directions of females and males as vectors from the abdomen tips to the head front. Then, a pair was in the female leader when the male was behind relative to the female heading direction, and the female was front relative to the male heading direction, and vice versa (Fig. 1AB). If a pair spent more than half of the time in a female-leader position during an interaction event, we regarded the interaction event as a female-leader tandem run. This classified all frames into female-leader tandem, male-leader tandem, other interactions (including tandem runs where they switch leader-follower roles), and non-interactions. We obtained the traveled distance for which the leader walked during each tandem running event. Then, we compared this traveled distance, using mixed-effects Cox models, with species being treated as a fixed effect and each pair id as a random effect. We used the coxme() function in the coxme package in R [46]. Note that we used distance instead of duration to evaluate how much tandem running pair could explore the environments by removing pausing time during interactions.

*Ancestral state reconstruction*

How we obtained the phylogeny is here.

Methods for ace here.

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**Table 1. Composition of reproductives in *G. fuscus* and *G. satsumensis*.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Mature colony |  |  |  |  |  |  |
| **Species** | **Colony** | **Location** | **PQ** | **PK** | **SQ** | **SK** |
| *Glyptotermes* *fuscus* | GPS253B | Ashizuri, Kochi | 0 | 1 | 1 | 0 |
|  | GPS329-1 | Toi, Miyazaki | 2 | 2 | 0 | 0 |
|  | GPS332 | Shibushi, Kagoshima | 2 | 1 | 0 | 0 |
|  | GPS313 | Issou, Yakushima, Kagoshima | 2 | 2 | 0 | 0 |
|  | GPS324 | Setouchi, Kagoshima | 2 | 2 | 0 | 0 |
|  | GPS063 | Setouchi, Kagoshima | 9 | 8 | 0 | 0 |
|  | GPS283-1 | Oku, Kunigami, Okinawa | 2 | 1 | 0 | 0 |
|  | GPS264 | Tanodake, Nago, Okinawa | 3 | 3 | 0 | 0 |
|  | GPS268 | Tanodake, Nago, Okinawa | 2 | 1 | 0 | 0 |
| *Glyptotermes* *satsumensis* | GPS253C | Ashizuri, Kochi | 2 | 3 | 0 | 0 |
|  |  |  |  |  |  |  |
| Incipient colony |  |  |  |  |  |  |
| **Species** | **Colony** | **Location** | **PQ** | **PK** | **SQ** | **SK** |
| *Glyptotermes* *fuscus* | GPS283-2 | Oku, Kunigami, Okinawa | 1 | 1 | 0 | 0 |
|  | GPS275 | Tanodake, Nago, Okinawa | 1 | 1 | 0 | 0 |
| *Glyptotermes* *satsumensis* | GPS329 | Toi, Miyazaki | 1 | 1 | 0 | 0 |
|  | GPS407-5 | Sata, Kagoshima | 1 | 1 | 0 | 0 |

PQ: primary queen, PK: primary king, SQ: secondary queen, SK: secondary king. Primary indicates alate derived. Secondary indicates neotenic.