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

**Nobuaki Mizumoto1\*, Simon Hellemans2**

1. Department of Entomology & Plant Pathology, Auburn University, Auburn, AL, 36849, USA

2. Okinawa Institute of Science & Technology Graduate University, Onna-son, Okinawa, 904-0495 Japan

\*: Correspondence: [nzm0095@auburn.edu](mailto:nzm0095@auburn.edu)

**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], and thus, nest establishment as a single termite is not usually successful [14,15]. Therefore, even if several termites have parthenogenetic ability [16,17], they need a partner for colony foundation. A species of drywood termite, *Glyptotermes nakajimai* Morimoto (Isoptera: Kalotermitidae) [18], 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) [19]. 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 [11,20], and female-female pairs start their nests with parthenogenesis [15,21], although they are not very successful in the long term [22–24]. 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 [25,26]) 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 [19]. 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 [18,28], 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.

**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 [30], while the sample from Cape Toi was asexual [19]. The field collection was performed before the swarming seasion; 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. Alates were then collected and separated individually. Tandem running behavior happens after termites shed their wings. We used individuals that shed their wings by themselves within 12 h.

Alates were then collected, separated by sex, and color-marked with one dot of paint (PX-20; Mitsubishi) on the abdomen to distinguish sex identities.

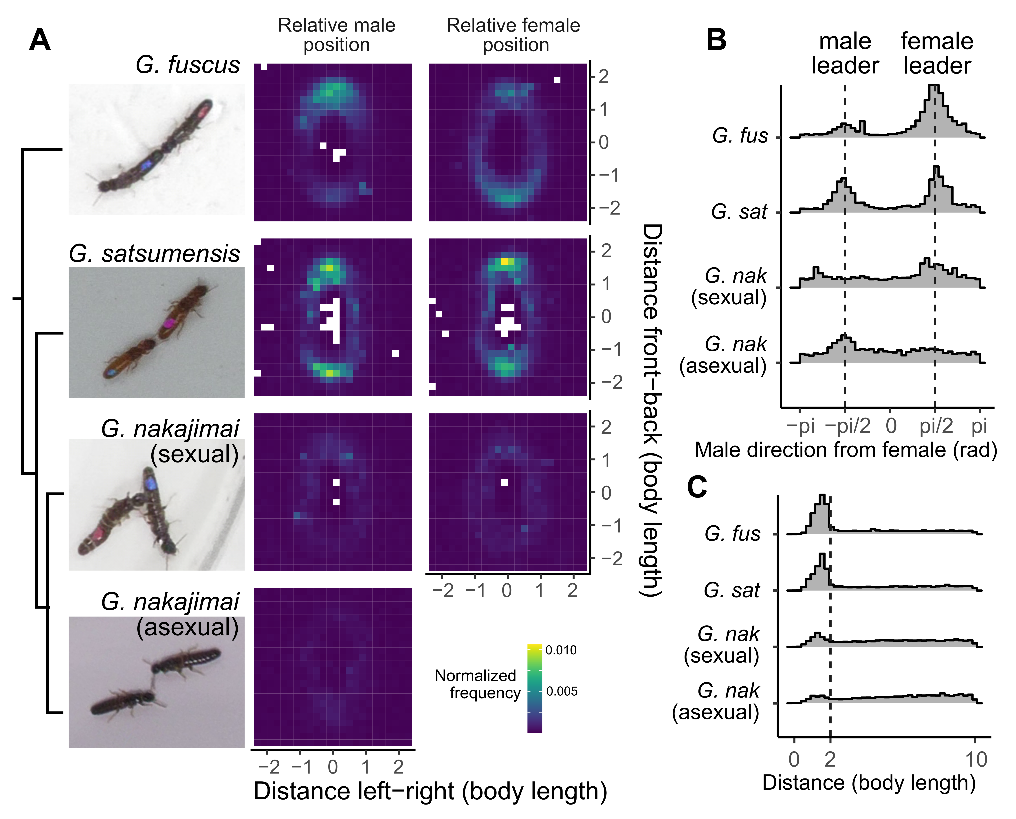
*Behavioral observations*

We introduced a female-male pair of termite dealates (female-female pair for 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. We recorded their behavior up to 60 minutes at the rate of 30 frames per second. All the videos were cropped to 1200x1200 pix to only include the arena in the frame before the video analysis. In total, we observed 21 pairs of *G. stsumensis* (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.

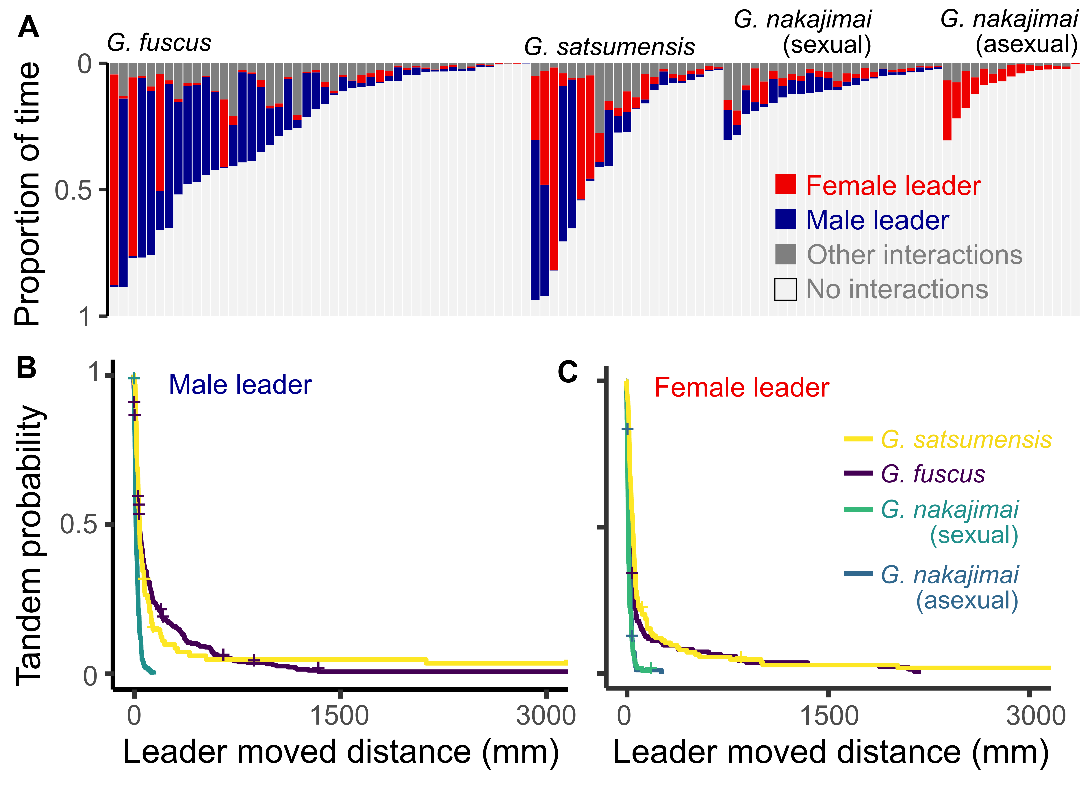
All videos were analyzed using SLEAP v 1.4.0 [29] to estimate the movement of body parts of each individual. We used a 6-node skeleton: antenna tips (LR), head (middle of mouth parts), head-pronotum boundary, body center, abdomen-tip, and a dot of color painted marker. We built a model for one species 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. In *G. fuscus*, TBA.

We used Python to format all HDF5 files for further analysis and converted them into FEATHER files for analysis in R [31]. 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 that pairs were in tandem or not based on the postures and spatial position of partners. First, we regarded two individuals were in interaction when the distance between body centers of partners was less than two body lengthes, 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 heading directions of females and males as vector from abdomen tips to head front. Then a pair was in female-leader when male was behind relative to female heading direction, and female was front relative to male heading direction, and vice versa (Fig. 1AB). If a pair spent in female-leader position for more than half of the time during an interaction event, we regarded that the interaction event was female-leader tandem runs. 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 obtrained the traveled distance for which the leader walked during each tandem running events. 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 coxme() function in the coxme package in R [32]. Note that we used distance instead of duration to evaluate how much tandem running pair could explore the envronments by removing pausing time during interactions.



**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 [19] 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.

**Idea of another result to be shown.**

I want to show that the colonies of G. nakajimai include many reproductives (not a pair) [19], while colonies of G. fuscus and G. satsumensis often have just monogamous pairs (often physogastric). This is consistent with my observation, but my data is minimal as I have not recorded it properly. The former is shown in the paper [19], but we do not have published information on the latter.

Here are some possible approaches:

Based on this project report (you can read this in English from this link: <https://koara.lib.keio.ac.jp/xoonips/modules/xoonips/detail.php?koara_id=2018000005-20180253>), G. satsumensis keeps monogamous pairing. However, this report is not published (or looks like will not be published in the future). We may reach out Hayashi-san to ask if he has any data about colony structures in G. satsumensis. If so, we can ask if he can join this paper.

We can also reach out Yashiro-san if he have data.

Do you have any thoughts/ideas?

**Idea of Discussion**

Although most theoretical studies of the evolution of sexuality have actually acknowledged that nonrandom mating or parental care may infuence the outcome of their models, the importance of these phenomena has always been minimized [7].

The resource limitations may have facilitated the evolutionary loss of tandem runs and pleometrosis [27]. The distribution of G. nakajimai is highly localized compared to G. fuscus or G. nakajimai. They are all one-piece nester.

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HATCH project number.

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