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

**Nobuaki Mizumoto1,2\*, Toshihisa Yashiro3,4,5, 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

3. Graduate School of Agriculture, Kyoto University, Kyoto, 606-8502, Japan

4. School of Life and Environmental Sciences, University of Sydney, Sydney, NSW, 2006, Australia

5. Present address: Koshi Research Station, Institute for Plant Protection, National Agriculture and Food Research Organization, Koshi 861-1192, Japan

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

**Abstract**

Parthenogenesis and the loss of males are reported from diverse organisms. Yet, asexually reproducing lineages are rare in social animals that exhibit biparental care, often as part of the sequence of dispersal, pairing, mating, and reproduction. The male-less lineages of the termite, *Glyptotermes nakajimai*, provide a unique opportunity to study how sexual reproduction can be lost in such animals. 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. However, tandem running was rare and ephemeral in both sexual and asexual lineages of *G. nakajimai*. Furthermore, our comparative studies across the entire termite diversity showed that the typical monogamous pairing was uniquely lost in *G. nakajimai*, while *G. fuscus* and *G. satsumensis* initiated nests in pairs. Our study evidence a clear disruption of the reproductive behavioral sequence in both sexual and asexual lineages of *G. nakajimai*, coupled with the evolution of an alternative mode of colony foundation. Our study highlights one evolutionary path to asexuality in social animals, retaining biparental care without the necessity of two sexes.

**Significance statement**

The evolution of unique animal societies involves modifications in physiological and behavioral phenotypes, distinguishing them from related lineages. Unlike social Hymenoptera, termites represent a bisexual insect society that stems from biparental care. In this study, we demonstrated the behavioral preadaptation towards the evolution of a male-less society in a termite, *Glyptotermes nakajimai*. Our combination of movement trajectory analysis with posture tracking and phylogenetic comparative analysis revealed that the loss of monogamous pair formation was a prerequisite to male-less societies. Hence, the evolutionary loss of males can occur in animals with biparental care, provided major disruption of their reproductive behavioral sequence.

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

**Introduction**

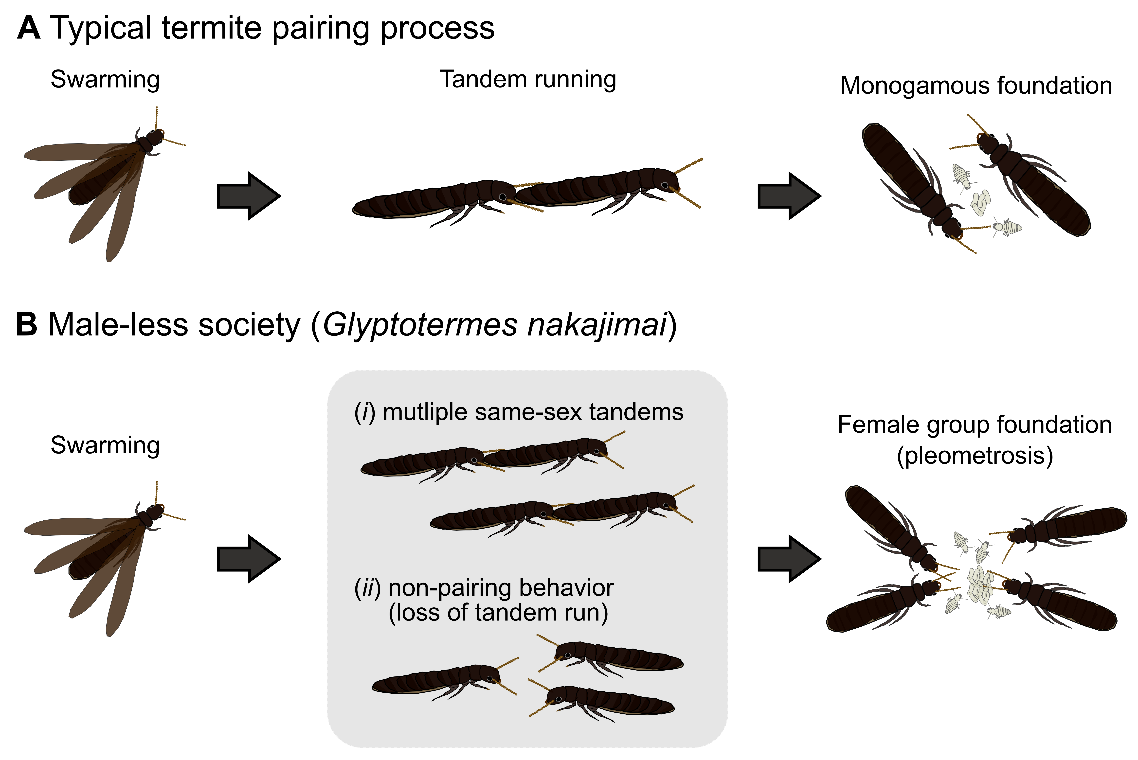
Despite the costs of producing males, sexual reproduction remains the norm among animals (1–3), and the evolution of fully asexual populations is rare due to developmental, genetic, and behavioral barriers (4, 5). These barriers are strong in social species with biparental care, as males contribute to their offspring beyond providing sperm (2, 6). Social Hymenoptera include many male-less lineages (7–9) as they rely solely on maternal care and have an asexual reproduction capacity due to haplodiploidy (10). In contrast, diplodiploid social animals often maintain a tight link between sexual reproduction and mate pairing, making males integral to their social systems through biparental care (11–13). This is especially evident in termites, the hallmark of non-Hymenopteran eusociality, which form bisexual societies initiated by a pair of a female and a male (14–16), where male-less societies are extremely rare.

Biparental care is a clear barrier hindering the evolution of male-less societies in termites. In the initial phases of colony foundation, males significantly contribute to the offspring energetically and through physical labor (17, 18). Many termite species possess the capacity of parthenogenesis or tychoparthenogenesis (occasional egg development without fertilization), without evolving into fully asexual lineages (19–21). Although such females could, in theory, reproduce alone, colony foundation by a single female is not successful, as they require a partner for allogrooming (22). Therefore, the transition to complete asexuality requires drastic modifications to their typical monogamous colony foundation.

The drywood termite, *Glyptotermes nakajimai* Morimoto (Isoptera: Kalotermitidae) (23, 24), offers a rare opportunity to study the evolution of asexuality in animals with biparental care. In several populations, males have been completely lost, resulting in fully asexual populations (25, 26). Distinct from typical monogamous termites (Fig. 1A), *G. nakajimai* colonies are often headed by multiple reproductives (25). Such pleometrosis (colony foundation by multiple kings and queens) could facilitate the evolution of an asexual society by securing sufficient partners without males. However, how these termites achieved such a modified colony foundation from a typical monogamous pairing remains unknown.

We hypothesized that the pleometrosis facilitated the evolution of asexuality in early lineages of *G. nakajimai*. In many termite species, after the dispersal flight, de-winged reproductives exhibit tandem courtship while searching for a nest site (27, 28). Two behavioral preadaptations could enable the male-less colony foundation with pleometrosis(Fig. 1B). First, same-sex (female-female) pairs may have provided an intermediate state (Fig. 1B-*i*). For example, *Reticulitermes* termites show stable female-female tandems (28, 29) and can initiate colonies via parthenogenesis but with limited long-term success (30, 31). If early lineages of *G. nakajimai* exhibit tandem runs, multiple same-sex tandems could have enabled pleometrosis without major behavioral shifts. Alternatively, termites might have lost typical tandem pairing so that they can achieve pleometrosis without pair formation (Fig. 1B-*ii*). In this scenario, *G. nakajimai* may have largely modified their ancestral pairing processes from other termite lineages.

To test these ideas, we studied three sympatric *Glyptotermes* species in Japan: *G. nakajimai, G. fuscus* Oshima*,* and *G. satsumensis* (Matsumura). They are phylogenetically closely related, but asexual lineages only arose in *G. nakajimai*. We quantified tandem running behavior using deep-leaning automated posture-tracking software (32) and described their colony breeding structures from the field observations. Furthermore, we reconstructed a mitogenome-based phylogenetic tree to unravel the evolution of tandem running behavior and mating systems across termite diversity. Altogether, our analyses shed light on the evolution of male-less societies through the lens of behavior.



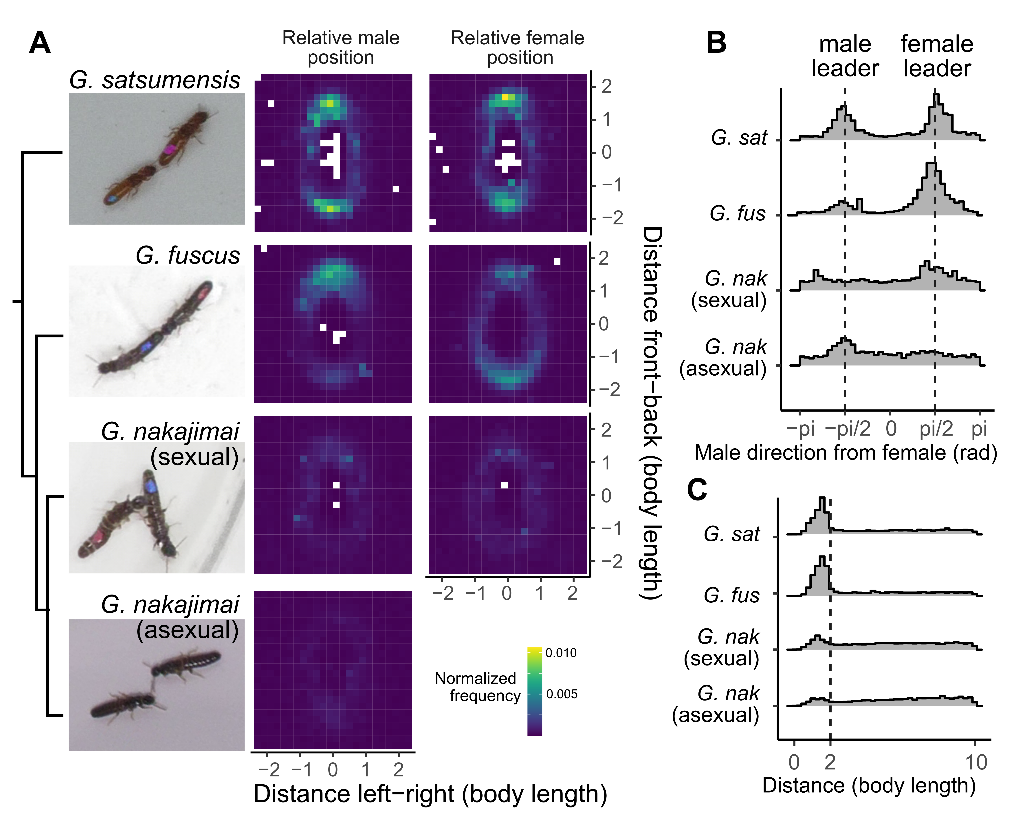
**Figure 1.** Comparison of (A) the typical termite mate pairing process and (B) potential behavioral changes for the evolution of a male-less society. Unlike monogamous pairing in typical termites, the male-less society of *G. nakajimai* is headed by multiple females. Two behavioral models could achieve this: (*i*) use of multiple same-sex tandem running, requiring minimum behavioral changes, and (*ii*) use of a non-pairing mechanism following the evolutionary loss of tandem running behavior.

**Results and Discussion**

*Lack of tandem pairing in G*. nakajimai

Our behavior experiments clearly highlighted tandem running in *G. fuscus* and *G. satsumensis*, while it was lacking in *G. nakajimai* from both sexual and asexual populations (Figs. 2 and 3). First, each species showed distinct patterns in pair positioning when we observed behavioral interactions of mating pairs (or female-female pairs in asexual populations) in a petri dish arena. When species-specific datasets were pooled, pairs were often found to be within two body length distances across all species, where data distribution seemed to follow different patterns within or beyond two body lengths (Fig. 2AC), indicating that termites dealates interact when within two body lengths.

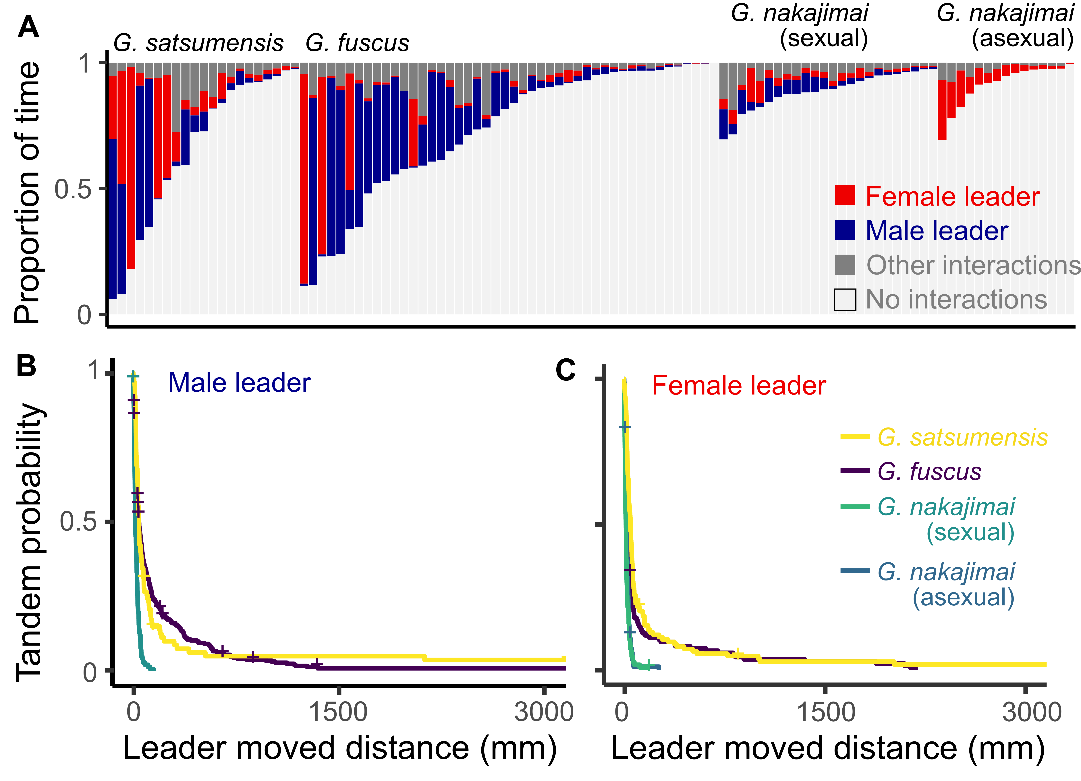
Second, when termites were within two body lengths, the spatial orientations of pairs differed across species. In *G. satsumensis*, pairs were often positioned in a line, with the female either in front or behind the male (Fig. 2AB) —*i.e.*, they exhibit both female-led and male-led tandems. In *G. fuscus*, tandems were more frequently male-led (Fig. 2AB). In contrast, we could not observe a clear trend for the positioning in *G. nakajimai*. This species exhibited neither leader nor follower in both sexual and asexual populations (Fig. 2AB). We used these observations to define 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).



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

Next, we looked into the details of the pairwise interactions for each pair. Tandem running behavior was highly variable across different pairs, even within species (Fig. 3A). Some pairs showed tandem runs during the entire observation period, while some did not form tandem runs. 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* (0/39). There was no significant difference in the proportion of time for tandem running behavior among species (GLMM, χ23 = 3.74, *P* = 0.291). Within each species, the proportion of time spent in female-led and male-led tandem runs was different: female-led tandem running was more common in *G. satsumensis* (GLMM, χ21 = 22.92, *P* < 0.001), while male-led tandems were more common 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 (i.e., the follower) for pairing in *G. satsumensis*, while the female is the more active sex for pairing in *G. fuscus* and *G. nakajimai*. Such an active role of females might have contributed to the evolutionary loss of males, as active males can prevent the evolution of asexuality (4).

The function of tandem running behavior is to maintain pair cohesion while exploring the environment for finding a nesting site. Therefore, it 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. Our results evidenced that *G. satsumensis* and *G. fuscus* traveled a longer distance while in a pair than *G. nakajimai* did (Fig. 3; Cox mixed effect model, male leader, χ23 = 30.19, *P* < 0.001, female leader, χ23 = 17.56, *P* < 0.001). Both lineages of *G. nakajimai* exhibited ephemeral tandem runs, with their longest tandem breaking up after running only 185 mm (sexual) and 260 mm (asexual), which challenges their functionality. On the other hand, tandem running of *G. satsumensis* and *G. fuscus* can travel for more than 1,000 mm long in 24 events (15: *G. fuscus*, 9: *G. satsumensis*, from 16 pairs, 11: *G. fuscus*, 5: *G. satsumensis*). Within these species, there were no significant distance 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). Thus, *G. nakajimai* has lost the typical tandem pairing compared to related species, which would have allowed them to use an alternative colony foundation method of pleometry (Fig. 1B-*ii*).



**Figure 3.** 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. Kaplan–Meier survival curves were generated for each species, and the symbol “+” indicates the right-censoring, where the tandem run had not ended by the end of observations.

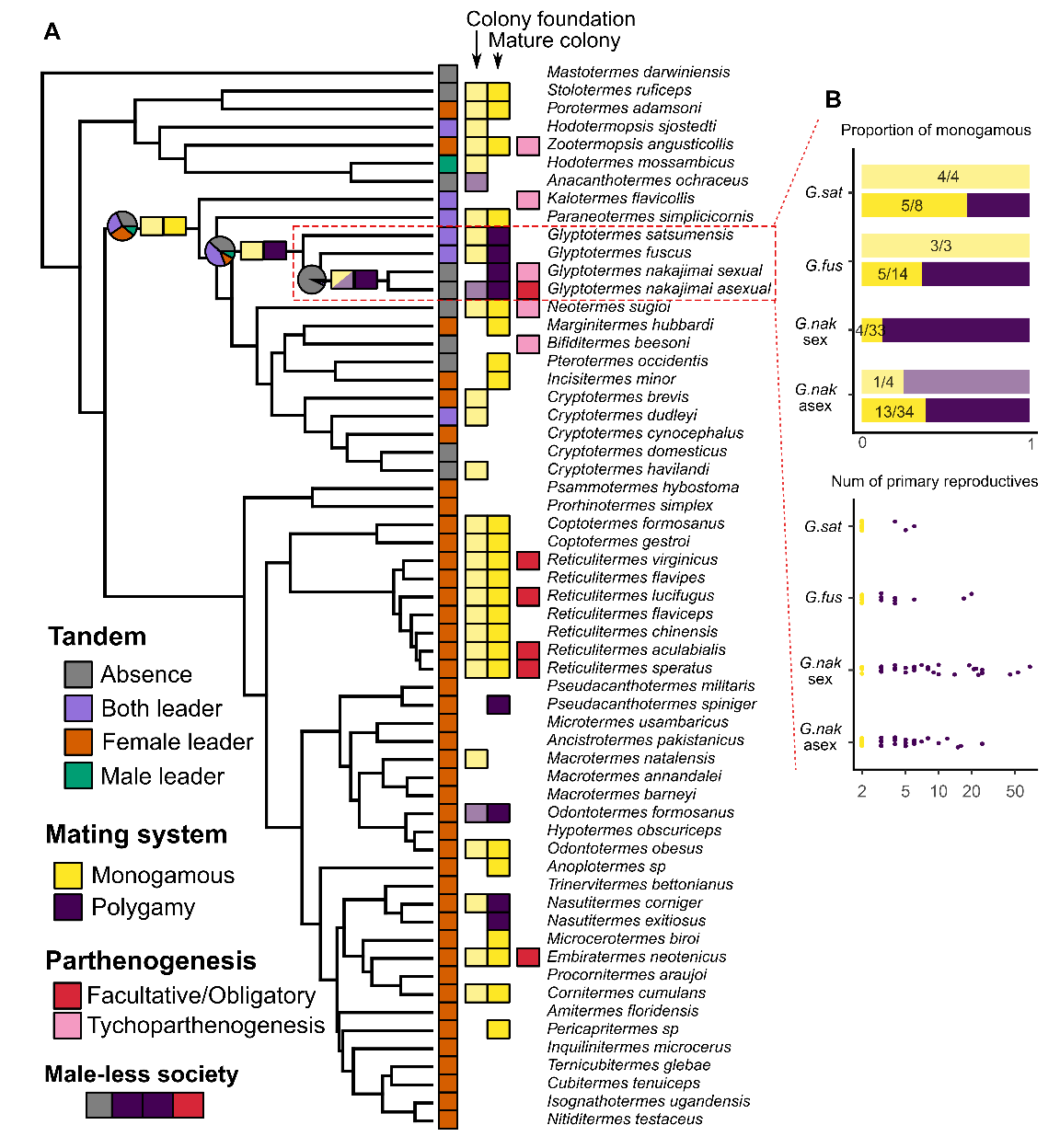
*Ancestral state of tandem running behavior*

Tandem running behavior is highly variable in Kalotermitidae termites (28). Genus-level ancestral state reconstruction and the fossil record suggested that the ancestor of Kalotermitidae exhibited tandem running (28, 33). Here, we evidenced that *G. nakajimai* does not show tandem running, while *G. fuscus* and *G. satsumensis* do (Figs. 2 and 3). These suggest two evolutionary processes of tandem running in termites: 1) tandem running behavior in Kalotermitidae exhibits greater evolvability than the other lineage, allowing for more frequent evolutionary changes; 2) this evolutionary flexibility may have enabled the loss of tandem pairing in *G. nakajimai*, even before the emergence of its male-less society. We tested these hypotheses in a comparative phylogenetic framework.

First, we reconstructed the relationships among species with known tandem running state from complete mitogenomes. Our analyses revealed that the sexual and asexual lineages of *G. nakajimai* diverged 14.47 million years ago (Mya) (95% confidence interval; CI95: 8.79–21.19 Mya), and exhibit a rather low similarity between lineages (91.19%). Our results are in line with previous results based on one mitochondrial gene *COII*: sequences exhibited a similarity of 91.67% (sexual: KX688853; asexual: KX688849), and estimated divergence of 14.1 Mya (CI95 = 8.1–22.4 Mya) among lineages (25). Our results, therefore, indicate that *G. nakajimai* is evolutionarily old. Whether these two lineages should be treated as distinct species remains open.

We performed the species-level ancestral state reconstruction using published datasets. By fitting various evolutionary models for tandem running evolution, we found that the evolutionary rate of tandem running behavior is distinct between Kalotermitidae (+ other basal lineages) and Neoisoptera, representing two major termite groups in their diversity (Figs. S1 and 4). The best-fit model was a hidden rate model (34), where the female-leader feature can have two distinct states (Fig. S1). (i) Although female-leader tandem running is observed in Kalotermitidae, the female leader was estimated as plastic and can readily switch to male-leader (Fig. S1). (ii) In contrast, female-leader tandems in Neoisoptera were estimated to be evolutionarily fixed (Fig. S1), likely through the evolution of conserved sex pheromones (35).

Our reconstructions could not ascertain the ancestral tandem running state of Kalotermitidae (Figs. S1 and 4): it lacked tandem running in 32% probability, while it could exhibit tandem running in 68% either as female leader (29%), male leader (11%), or both leader (28%). Note that this information may be conservative, as our estimation lacks fossil evidence of tandem running of *Electrotermes affinis*, an extinct kalotermitid termite with tandem running behavior (33). Similarly, the ancestral state of Japanese *Glyptotermes* was also ambiguous but more likely doing tandems (Figs. S1 and 4). The ancestral state was estimated as lacking tandem running at 38%, while exhibiting tandem running at 62% either as female leader (11%), male leader (2%), or both leader (42%). However, our analyses evidenced that the ancestral lineages of *G. nakajimai* clearly lacked tandem running behavior in 93% (Figs. S1 and 4). Therefore, we conclude that the loss of tandem pairing should have predated the evolution of a male-less society in *G. nakajimai* (Fig. 1 *ii*).



**Figure 4.** Combination of behavioral phenotypes towards the evolution of a male-less society. (A) The evolution of tandem running behavior with relationship to mating systems in termites. The information on tandem running is from (28), mating system from Table S1-2, and parthenogenesis from (20, 36). The results of ancestral state reconstruction were shown for a few nodes towards the evolution of a male-less society. Note that we did not perform reconstruction for parthenogenesis because many species lack information about “lacking parthenogenesis.” (B) Comparison of breeding systems in *Glyptotermes* termites.

*Mating system evolution for a male-less society*

Finally, to investigate how pleometrosis (or having multiple primary reproductives) is distributed among termite diversity and contributes to the evolution of a male-less society, we performed phylogenetic comparative analyses of the mating systems (Fig. 4A). We compiled information about the number of reproductives in the nest for the species with the available tandem running information by performing a systematic search of the literature (Table S2). Furthermore, we report new field data on the colony structure of *G. fuscus* and *G. satsumensis* (Table S1).

In *Glyptotermes*, mature colonies of *G. fuscus* and *G. satsumensis* often included multiple reproductives (Table S1, Fig. 4B). The total number of reproductives was variable across species (GLM, χ23 = 188.7, *P* < 0.001), where sexual populations of *G. nakajimai* had more reproductives than others (Tukey’s posthoc *P* < 0.001), and there was no significant difference among asexual populations of *G. nakajimai,* *G. fuscus,* and *G. satsumensis* (Tukey’s posthoc, *P* > 0.09). However, the source of such multiple reproductives in mature colonies differed among species. Incipient colonies of *G. nakajimai* already had multiple queens (25), suggesting colony foundation via pleometrosis. In contrast, incipient colonies of *G. fuscus* or *G. satsumensis* were consistently monogamous pairs (pair: 3/3 in *G. fuscus* and 4/4 in *G. satsumensis*, Table S1, Fig. 4B). The number of reproductives increased from incipient to mature colonies in *G. fuscus* (GLM, χ21 = 6.93, *P* = 0.008), but not in *G. satsumensis* (GLM, χ21 = 1.3, *P* = 0.255), suggesting colony fusion as a potential source of additional reproductives. Notably, while colony fusion often leads to conflict and pair formation in many other species (37–39), *Glyptotermes* termites might be tolerant to the presence of other reproductives, which may have played a role in facilitating the evolution of pleometrosis in this group.

Our observations in *Glyptotermes* species are further corroborated by a large-scale comparative analysis (Fig. 4A and Table S2). Across termites, having multiple reproductives in the colony is rare. Pleometrosis has been observed in several species of Termitidae (40–42), where both monogamy and pleometrosis can be observed in a mixed way (Table S2). In Kalotermitidae, primary reproductives were usually found in monogamous pairs (43, 44), and the ancestral state was estimated as monogamous in both the colony foundation stage (89%) and mature colonies (98%; Fig. 4A). This is true even for the species that lacks tandem running and shows facultative parthenogenesis (i.e., *Neotermes sugioi*) (45, 46). On the other hand, *Glyptotermes* stands out with the ancestral state estimated to have multiple primary reproductives in mature colonies (94%, Fig. 4) but not in the incipient colony (11%). The combination of characteristics was unique in *G. nakajimai*, which lacked tandem running, started colonies from multiple reproductives and had the ability of parthenogenesis (Fig. 4A). In this group, the modification of pair formation (lack of tandem run and pleometrosis) should have predated the male-less society (Figs 1 and 4).

Lack of tandem running should be the prerequisite for pleometrosis. Tandem running behavior is essentially a pairing behavior between two individuals, and runs with more than three individuals are unstable (28, 29, 47). For example, in Macrotermitinae termites (e.g., *Odontotermes* or *Macrotermes*), it is relatively common to find colonies with more than two primary reproductives (40) (Table S2). Still, in these species, monogamous pairing is also common. However, we found that lack of tandem running does not always lead to pleometrosis, as several species lack tandem runs, yet remain restricted to monogamy. As another ecological component, resource limitation should facilitate pleometrosis (48). When the nesting site is a limited and valuable resource, termites must concentrate and be forced to start a colony with many individuals. The distribution of *G. nakajimai* is highly localized; this species is usually rarer than other species and can be found within the narrow area near the tips of the capes (especially in asexual populations). The particular requirements of a site of colony foundation should have contributed to the pleometrosis observed in *G. nakajimai*.

***Conclusion***

Sexual reproduction is the norm in the animal kingdom, and asexuality did not evolve broadly because each group has a specific, unique hurdle to overcome during male loss (3, 5). Many theoretical studies of the evolution of sexuality have acknowledged that parental care by males can minimize the cost of sexual reproduction. While parthenogenesis ability has evolved independently many times in termites (20, 21, 36), a complete asexual society only happens in *G. nakajimai* as far as we know. Using an integrative behavioral and phylogenetic framework, we showed that mate pairing behavior went through several steps, loss of tandem running and pleometrosis, all of which ultimately led to the loss of males. This highlights the importance of behavioral preadaptation as a prerequisite for the evolution of asexuality in social animals.

**Methods**

*Termite collection*

We collected three colonies of *G. fuscus* in Okinawa Prefecture (one in January 2021 and one in March 2022 in Nago; one in March 2023, Iriomote Is.), 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). The populations of *G. nakajimai* from Fukui prefecture and Tokunoshima Island are sexually reproducing (49), while the Cape Toi population is asexually reproducing (25).

The field collection was performed before the swarming season in March-June, depending on the location (23, 49); 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 the nests to 27 °C to trigger the swarming of alates. Nests were also opened with axes to promote dispersal and collect flying alates. Alates were induced to shed their wings by manually pinching their wings with forceps. Dealates were maintained in Petri dishes containing moist, unwoven cloth in the light and used for the experiments within one day of flight. Dealates were separated by sex and color-marked on the abdomen with one dot of paint (PX-20; Mitsubishi; color red = females; color blue = males).

*Behavioral observations*

We introduced a female-male pair of termite dealates (female-female pair for the asexual population of *G. nakajimai*) to the experimental arena, consisting of a petri dish (φ = 90 mm) covered with a layer of moistened plaster. We consistently used nest-mate pairing for valid cross-species comparisons. Note that although tandem running does not differ between nestmate and non-nestmate pairing in *Reticulitermes speratus* (28), the effect of intracolonial and intercolonial pairing on tandem running behavior is not known for *Glyptotermes* species. We recorded their behavior for up to 60 minutes at 30 frames per second. Videos were cropped to 1200x1200 pixels 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 (32) 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 the painted marker dot. We built a model for one species and then used it as a starting point to build another for the next species sequentially, rather than using one generic model, to improve the tracking accuracy. 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. Second, 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. Finally, the models for both sexual and asexual populations of *G. nakajimai* were trained based on the *G. fuscus* model. The models of *G. nakajimai* were developed for each colony separately to improve the tracking accuracy with small variation in recording conditions and body sizes. This approach allowed us to build on pre-existing models and fine-tune them, rather than starting from scratch, which reduced the time and resources needed for training.

We used Python to format all HDF5 files for further analysis and converted them into FEATHER files for analysis in R (50). 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 considered 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 the 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. A pair was defined as female-led when the male was behind relative to the female heading direction and the female was in front relative to the male heading direction; and vice versa to define a pair as male-led (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 timepoints into female-leader tandem, male-leader tandem, other interactions (including tandem runs where individuals switch leader-follower roles), and non-interactions. We obtained the traveled distance for which the leader walked during each tandem running event. Traveled distances were compared 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 (51). Note that we used distance instead of duration to evaluate the extent with which pairs could explore the environment by removing pausing time during interactions.

*Phylogenetic reconstructions*

We reconstructed a calibrated phylogenetic tree using mitochondrial genomes to carry out subsequent ancestral reconstructions of tandem states. We selected species with a focus on species and lineages with reviewed data on tandem runs (28), while also encompassing all recognized extant families and subfamilies *sensu* Hellemans *et al*. (52). Most mitochondrial genomes used in this study have been previously published (Table S3).

We sequenced six additional samples from species with data on tandem runs (Table S3). Specimens were collected in Japan and preserved in absolute ethanol, stored at -20°C until DNA extraction. DNA was extracted using the DNeasy Blood & Tissue extraction kit (Qiagen). Libraries were prepared using the NEBNext® Ultra™ II FS DNA Library Preparation Kit (New England Biolabs) and the Unique Dual Indexing Kit (New England Biolabs), with reagent volumes reduced to one-fifteenth of the recommended volumes. Libraries were pooled in equimolar concentration and paired-end sequenced on the Novaseq platform at a read length of 150 bp. Raw reads were trimmed from adapters and low-quality bases using fastp *v*0.20.1 (53). Trimmed reads were assembled using metaSPAdes *v*3.13 (54), and mitochondrial scaffolds were identified and annotated using MitoFinder *v*1.4 (55). The six mitogenomes produced herein were deposited in GenBank under accessions XXX-XXX.

Annotated mitochondrial features were separately aligned. For the 13 mitochondrial protein-coding genes, we translated DNA sequences into the corresponding amino acid sequences using the transeq function from EMBOSS *v*6.6.0 (56) and aligned protein sequences with MAFFT *v*7.305 (57). Protein alignments were back-translated into codon alignments using PAL2NAL *v*14 (58). The two rRNA and 22 tRNA mitochondrial genes were aligned as DNA sequences. All alignments were concatenated in a supermatrix using FASconCAT-G\_v1.04.pl (59). The mitochondrial supermatrix was separated into five distinct partitions: combined rRNAs, combined tRNAs, and combined first, second, and third codon positions of protein-coding genes.

Time-calibrated phylogenetic tree without the third codon positions partition was reconstructed using BEAST v2.6.2 (60). Trees and clock models were set as linked, while site models were kept unlinked. A GTR+G model of nucleotide substitution was selected for all partitions. The trees were given a Yule speciation process as prior. An uncorrelated lognormal relaxed clock was used to model rate variation among branches (61). We used 12 fossils as minimum age constraints (Table S4). Fossil calibrations were implemented as exponential priors on node time with a 97.5% soft maximum bound (62). Minimum age constraints and soft maximum bounds were obtained from PaleoBioDB (<https://paleobiodb.org>; last accessed: 25/02/12). We applied topological constraints to match the topology retrieved by genome-scale reconstructions (52, 63, 64). Specifically, we enforced: the (i) sistership of Stylotermitidae to all extant Neoisoptera; and the monophylies of (ii) Macrotermitinae + Sphaerotermitinae, (iii) non-Macrotermitinae non-Sphaerotermitinae Termitidae, (iv) Cylindrotermitinae, (v) Syntermitinae, and (vi) Amitermitinae+Promirotermitinae+Mirocapritermitinae+Cubitermitinae+Termitinae. The last three constraints were applied to avoid incongruent topologies of Termitidae subfamilies due to their subsampling. Markov Chain Monte Carlo analyses were run for a total of 500 million generations. Trees and parameters were sampled every 50,000 steps. We visually inspected the trace file with Tracer v1.7 (65) and accordingly used a burn-in of 20%. The maximum clade credibility tree with median heights was obtained using TreeAnnotator.

*Ancestral state reconstruction of tandem running*

In this study, we extended the previous genus-level reconstruction of tandem running behavior (28), to consider species-level patterns of tandem running behaviour in *Glyptotermes*. We used the previously constructed dataset (28), with several modifications. Namely, (i) we added new data from *Glyptotermes* species reported herein (Figs. 2 and 3). (ii) We included *Hodotermopsis sjostedti*, which exhibited tandem running with both females and males being leaders (66). (iii) We treated *Mastotermes darwiniensis* as not exhibiting tandem running as reported in the observation on this species is based on the observation of workers. Also, no record exists on tandem running, even though the throughout field observations (67). Note that treating this species as tandem running with both female and male leaders did not change our conclusions. (iv) Finally, some species with tandem running lacked information on the leader state in (28). We fixed a female-leader state for neoisopteran termites with missing data, as all studied species exhibit female-leader tandem runs. By contrast, we arbitrarily set the both-leader state for non-neoisopteran termites. Note that treating these as female-leader tandem running (which is more common in termites in general) did not change our conclusions.

We fitted a variety of models to examine the most feasible evolutionary scenario. We carried out ancestral state reconstructions by coding tandem running behavior in four different states: without tandem running, female-leader tandem running, male-leader tandem running, and tandem running with both sexes being a leader. Another potential approach is to consider the two different characteristics of female and male leaders. However, our preliminary analysis with the function fitPagel() shows that these two characters are dependent (*P* = 0.005). Hence, the combination of these two states can be regarded as the same as the four-state model.

First, we fitted simple models (without any hidden states), and with different state transition patterns: all rates different model (‘ARD’ model) to allow independent estimates for transitions to and from the three states; the all rates equal model (‘ER’ model), and a model assuming symmetrical transition rates between states (“SYM” model’) to estimate the ancestral state of this discrete trait. We used the fitMk() function (pi = fitzjohn) of the R package “phytools” v2.4.4 (68).

Second, we used a hidden rate model, assuming that the female-leader state has a hidden state (34), where the termite loses evolutionary variability. We adopted this approach because neoisopteran termites exhibit female-leader tandem runs, likely driven by female-specific sex pheromone (35) that may make the evolutionary change of the sexual leader role less likely. By contrast, tandem running is variable in other lineages (28), where sex roles can easily change, and tandem behaviour can even be lost, as they do not have sex-specific pheromones (35). We developed two hidden rate models, one with only female leader tandem having hidden states and one with all traits having hidden states. We used the ER model for the pattern of state transition, and the function fitHRM (pi = fitzjohn) of the “phytools” package. We used the Akaike information criterion (AIC) to compare model fits, and Akaike weights to evaluate the relative support for each model.

*Ancestral state reconstruction of mating systems*

To relate the evolutionary process of tandem running with colony foundation strategies, we performed a comparative analysis of mating systems. We investigated whether colonies of each termite species have a monogamous pair (a king and a queen) or multiple reproductives at two stages: during colony foundation (or incipient colony) and mature colonies. In termite colonies, there are two types of reproductives: alate-derived adult reproductives (primary queen and king) and neotenic (supplementary) reproductives that differentiate from the offspring of colony founders. Note that there are several exceptional examples of adultoids, which are supplemental reproductives derived from alates (69). Therefore, multiple reproductives in mature colonies can either reflect pleometrosis (colony foundation by multiple reproductives), colony fusion, or supplementary adultoids.

We conducted a literature survey on the species for which information on tandem running is available (28). On the 15th and 16th of March 2025, we used the online reference platform Google Scholar with the simple string function and search strings: (Genus-name Species-name) AND (reproductives OR king OR queen OR foundation). We used Google Scholar to collect as much information on the number of reproductives as possible. We focused on the field observations reporting the number of alate-based (or primarily) reproductives in the incipient or mature colony. We also included indirect information based on the genetic marker of workers if the conclusion was a monogamous pairing (e.g., (70)). This is because the estimation of potentially multiple reproductives from genetic markers cannot rule out the possibility that workers from different colonies mixed in the foraging area, without having multiple reproductives at the same place (71). We also included the behavioral observation of colony foundation behavior, where some described the behaviors facilitating monogamy, such as aggression towards other pairs or sealing the nest entrance (e.g., (72, 73)). When most information was one type (monogamous or multiple reproductives) and the other was rare or exceptional, we categorized the species as the majority type. On the other hand, if both modes were observed equally, we recorded the species as multiple reproductives. All information is summarized in Table S2.

In addition, we specifically recorded new data on the species of *Glyptotermes* for this study. We obtained the number of reproductives of *G. fuscus* and *G. satsumensis* from field observations using the previously described method on *G. nakajimai* (25). Three incipient and 15 mature colonies of *G*. *fuscus* and four incipient and nine mature colonies of *G*. *satsumensis* were collected from Japan, in: Shikoku, Kyushu, Amami-Oshima Island, Okinawa Island, and Ogasawara Islands (Table S1). The nest woods were dismantled, and all colony members, including queens and kings, were extracted using an aspirator and forceps.

We performed ancestral state reconstructions of the mating system, separately for the incipient and mature colonies. We discarded species for which data could not be obtained. We used a method similar to the reconstruction of tandem running behavior described above. In both cases, a simple ER model was the best fit over others.

**Acknowledgments**

We thank Kensei Kikuchi, Esra Kaymak, Dr. Kazuya Kobayashi, Dr. Yasushi Miyaguni, Dr. Tomonari Nozaki, Yusuke Namba, and Manabu Yashiro for field collection, Aoi Mizumoto for assisting in video formatting, Dr. Thomas Bourguignon for lab space, and Dr. Kenji Matsuura and Dr. Nathan Lo for the support during the early stages of this work, and members of the Mizumoto lab at Auburn University for helpful discussion. We thank the Sequencing Section (SQC) and the Scientific Computing & Data Analysis Section (SCDA) of OIST for assistance with sequencing and providing access to the OIST computing cluster, respectively. We acknowledge the use of ChatGPT, a language model developed by OpenAI, for minor suggestions with respect to the texts and code. The work was supported by a JSPS Research Fellowship for Young Scientists CPD (20J00660), a Grant-in-Aid for Early-Career Scientists (21K15168), USDA National Institute of Food and Agriculture, Hatch project number 7007938 to NM.

**Author contributions**

N.M.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, visualization, writing-original draft

T.Y.: investigation, resources, writing-review and edit

S.H.: conceptualization, data curation, formal analysis, investigation, methodology, validation, supervision, writing-review and edit

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