



Ancestral sex-role plasticity facilitates the evolution of same-sex sexual behavior

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Edited by Raghavendra Gadagkar, Indian Institute of Science, Bangalore, India; received July 19, 2022; accepted September 29, 2022

Recent attempts to explain the evolutionary prevalence of same-sex sexual behavior (SSB) have focused on the role of indiscriminate mating. However, in many cases, SSB may be more complex than simple mistaken identity, instead involving mutual interactions and successful pairing between partners who can detect each other's sex. Behavioral plasticity is essential for the expression of SSB in such circumstances. To test behavioral plasticity's role in the evolution of SSB, we used termites to study how females and males modify their behavior in same-sex versus heterosexual pairs. Male termites follow females in paired "tandems" before mating, and movement patterns are sexually dimorphic. Previous studies observed that adaptive same-sex tandems also occur in both sexes. Here we found that stable same-sex tandems are achieved by behavioral plasticity when one partner adopts the other sex's movements, resulting in behavioral dimorphism. Simulations based on empirically obtained parameters indicated that this socially cued plasticity contributes to pair maintenance, because dimorphic movements improve reunion success upon accidental separation. A systematic literature survey and phylogenetic comparative analysis suggest that the ancestors of modern termites lack consistent sex roles during pairing, indicating that plasticity is inherited from the ancestor. Socioenvironmental induction of ancestral behavioral potential may be of widespread importance to the expression of SSB. Our findings challenge recent arguments for a prominent role of indiscriminate mating behavior in the evolutionary origin and maintenance of SSB across diverse taxa.

behavioral plasticity | collective behavior | leadership | same-sex sexual behavior | tandem runs

Same-sex sexual behavior (SSB) is widespread in nonhuman animals, and its evolution has attracted research interest because of its assumed fitness costs (1–4). Recent theory has suggested that selection against SSB may be constrained by costs of perfect sex recognition, thus maintaining SSB at low levels (5), and ancestral indiscriminate mating has been suggested to underlie the evolutionary origins of SSB (6–8). However, these arguments overlook an important consideration. SSB is often more than misdirected behavior otherwise expressed during heterosexual encounters. In many species, sexual interactions between same-sex partners involve at least one partner expressing behaviors associated with the other sex, such as mimicking the other sex (9) or expressing the other sex's role during pair bonding (10), and evidence is emerging that SSB can involve distinct behavioral repertoires with separate neurological causation (11). The sex of the partner in a same-sex pairing is different from that in a heterosexual pairing, so plastic responses to different sociosexual environments should be essential for the expression of SSB. Here we use a well-characterized insect system to test whether and how such sex-role plasticity facilitates the evolution of SSB.

In the termite *Reticulitermes speratus*, life-long monogamous pairs establish colonies and produce thousands of offspring (12). During a brief period, alates (winged adults) disperse from their nests. Both females and males land on the ground, shed their wings, and run to search for a mating partner (13). Upon joining, a pair performs a tandem run. The male follows the female, maintaining contact in a highly coordinated manner while seeking a suitable site for colony foundation. As soon as they find a suitable site, pairs establish a nest, and thus tandem pairing is not a temporary relationship but leads to a long-term pairing. Tandem running involves communication via sex pheromones (14), and all mating pairs engage in this process (15). For these reasons, tandem running is considered a form of extended courtship (13) or sexual behavior (16). A stable tandem run is maintained via two behavioral processes. First, bidirectional feedbacks between females and males enable them to actively regulate movement speed according to partner distance (17). Second, sexually dimorphic movements are expressed upon accidental separation, where females pause and males engage in an intensive search to facilitate reencounter (18).

Significance

The evolution of same-sex sexual behavior (SSB) is an enigma because such behavior cannot directly result in reproduction. Theoretical papers predict that indiscriminate mating is an evolutionary driver of SSB, calling for empirical work to test this hypothesis. Here we show that same-sex pairing in termites is maintained not by indiscriminate mating but by behavioral plasticity with accurate sexual discrimination. Female and male termites can express the behavior of the other sex, which contributes to maintaining pair coordination. Phylogenetic comparative analysis suggests that such behavioral flexibility was inherited from an ancestral lineage. We show that SSB can evolve with highly accurate sex discrimination, combined with sex-role plasticity.

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Author contributions: N.M. and N.W.B. designed research; N.M. performed research; N.M. contributed new reagents/analytic tools; N.M. analyzed data; and N.M., T.B., and N.W.B. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at <http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2212401119/-DCSupplemental>.

Published November 8, 2022.

Previous work has shown that same-sex pairing and tandem runs occur in *R. speratus* (19) and can increase fitness when heterosexual partners are unavailable during the critical dispersal period. The adaptive benefits of same-sex pairing arise via various mechanisms that permit survival long enough to achieve mating with individuals of the other sex later. For example, same-sex tandems dilute risk from predators that capture a single prey at a time, such that individuals can swap partners to form a heterosexual pairing upon encountering the opposite sex (19, 20). Same-sex pairs can also establish long-term (>1 y) nests, which facilitate survival (21). Nest establishment by a single individual is less successful (22) or impossible (21) because termites cannot self-groom their entire body and require a grooming partner (23). After nest establishment, female–female pairs can reproduce via parthenogenesis (23, 24) (*SI Appendix, Text S6*), and male–male pairs can invade neighboring incipient colonies to gain reproductive opportunities (21). Same-sex pairs establish nests as rapidly as heterosexual pairs (21), and nests with same-sex pairs have been observed in (semi)natural conditions (25, 26). Thus, while the highest fitness option for termites is to establish a heterosexual pair and nest, same-sex pairings function to “make the best of a bad job” by increasing the likelihood of nonzero reproductive fitness (21).

We combined experimental work on *R. speratus* with individual-based simulations and systematic comparative analysis to test the hypothesis that ancestral plasticity in sex roles potentiated the evolution of SSB. During same-sex tandem runs, at least one individual must express behaviors associated with a different role than they would express in a heterosexual pair. Thus, we predicted that behavioral plasticity in response to the partner is critical for stable same-sex tandems. Using detailed behavioral analyses of each partner during heterosexual and same-sex pairing, we show that individuals in same-sex tandems plastically modify their movements to achieve dimorphic behavioral processes similar to that of heterosexual tandems. We then used simulations based on movement parameters obtained through empirical observation to evaluate the contribution of sex-role plasticity in maintaining SSB, by comparing scenarios with and without plasticity. Finally, we determined the phylogenetic origin of sex-role plasticity using a comparative approach. Our findings illustrate how socioenvironmental induction of ancestral plasticity can facilitate the evolution of SSB.

Results

Phenotypic Plasticity of Sexually Dimorphic Behavior Enables Same-Sex Tandem Runs. We first evaluated the overall tendency for the formation of different tandem run combinations in *R. speratus*. Alates were collected in March 2021 from Kagoshima, Miyazaki, and Fukui prefectures, Japan. After swarming, we allowed individuals to freely interact in mixed or single-sex groups composed of individuals randomly selected from the same colony. Termites from the same colony are the offspring of the same king and queen. Thus, we used a full-sibling split-family experimental design, ensuring that any differences in tandem running behavior reflected plastic responses to the sociosexual environment experienced by each individual in a pair (i.e., sex or behavioral role of the partner), as opposed to genetic differences. Within these groups, female–male (FM) and male–male (MM) tandems occurred more commonly than female–female (FF) tandems. More individuals engaged in tandem runs in mixed groups or male-only groups than in female-only groups (generalized linear mixed model [GLMM], $\chi^2_2 = 16.151$, $P < 0.001$; Tukey honestly significant difference, FM–FF: $P = 0.010$, effect size [d] = 1.456, MM–FF: $P < 0.001$, effect size [d] = 1.870, FM–MM: effect size [d] = 0.468, $P = 0.617$) (*SI Appendix,*

Fig. S1). In mixed groups, we observed 27 heterosexual tandems and one male–male and one female–female tandem (see also *SI Appendix, Text S1* for results from an experiment with larger mixed groups, in which we observed 166 female–male tandems, 36 male–male tandems, and one female–female tandem).

Next, we moved a tandem pair from each group to a separate arena to further observe their behavior. Once a pair formed, same-sex tandems were as stable as heterosexual tandems, with no difference in the time spent in tandem during 5-min observations across pairing combinations (linear mixed model [LMM], $\chi^2_2 = 1.868$, $P = 0.396$) (*SI Appendix, Fig. S2A*). Same-sex pairs showed behavioral patterns strikingly similar to opposite-sex pairs. For example, leaders and followers in same-sex tandems regulated their motion acceleration in response to changes in interindividual distances (*SI Appendix, Fig. S3*). When interindividual distance increased, the follower sped up while the leader slowed down to avoid separation. When interindividual distance decreased, the follower slowed down while the leader sped up to avoid collision. The leader–follower role was fixed within a pair. In female–male or female–female pairs, we observed no switch of leader–follower roles (0 of 54 for female–male, 0 of 45 for female–female pairs), indicating no competition over position. In contrast, in male–male pairs both males often tried to follow each other, forming circles (competition >5 s observed in 11 of 54 pairs) (*Movie S1*). However, this rarely resulted in a switch of leader–follower positions (1 of 54 pairs), consistent with a previous observation that larger males usually took the follower position after forming circles (19).

During tandem runs, leaders and followers strongly synchronized their movements with one another, with no significant difference in movement speeds in any pairing combination (LMM, $P > 0.05$) (Fig. 1 *A, B, D, and F* and *SI Appendix, Fig. S4A*). However, when pairs became accidentally separated, leaders and followers showed distinct movements. In heterosexual tandems that became separated, leader females paused while follower males continued moving, consistent with previous findings (18). Thus, movement speed following separation differed notably between leaders and followers in heterosexual pairs (LMM, $\chi^2_1 = 41.246$, $P < 0.001$, effect size [d] = 1.764) (Fig. 1 *A* and *C* and *SI Appendix, Fig. S4B*). We found similarly distinctive dimorphic movement patterns in separated same-sex tandems: movement patterns were dictated by leader–follower position, not sex, where movement speed of the follower was always faster than that of the leader (female–female pairs: LMM, $\chi^2_1 = 60.471$, $P < 0.001$, effect size [d] = 2.345, male–male pairs: LMM, $\chi^2_1 = 71.077$, $P < 0.001$, effect size [d] = 2.295) (Fig. 1 *E* and *G* and *SI Appendix, Fig. S4B*). For example, in female–female tandems, leader females slowed down in the manner of females of heterosexual tandems, but follower females kept moving to search for the stray partner in the manner of males in heterosexual tandems. Similarly, in separated male–male tandems, follower males moved to search for the stray partner while leader males paused. Accordingly, individuals in same-sex pairs reencountered separated partners as effectively as in heterosexual pairs. By observing spontaneous separation events during tandem observations, we found that the time required for a pair to reencounter was not different between pairing combinations (mixed-effects Cox model, $\chi^2_2 = 3.203$, $P = 0.202$) (*SI Appendix, Fig. S5*).

Male and female same-sex pairings did not generate identical behavioral dynamics, however. After separation, females in female–female pairs moved slower than males in male–male pairs (LMM, follower: $\chi^2_1 = 7.454$, $P = 0.006$, effect size [d] = 0.780; leader: $\chi^2_1 = 10.04$, $P = 0.002$, effect size [d] = 0.905)

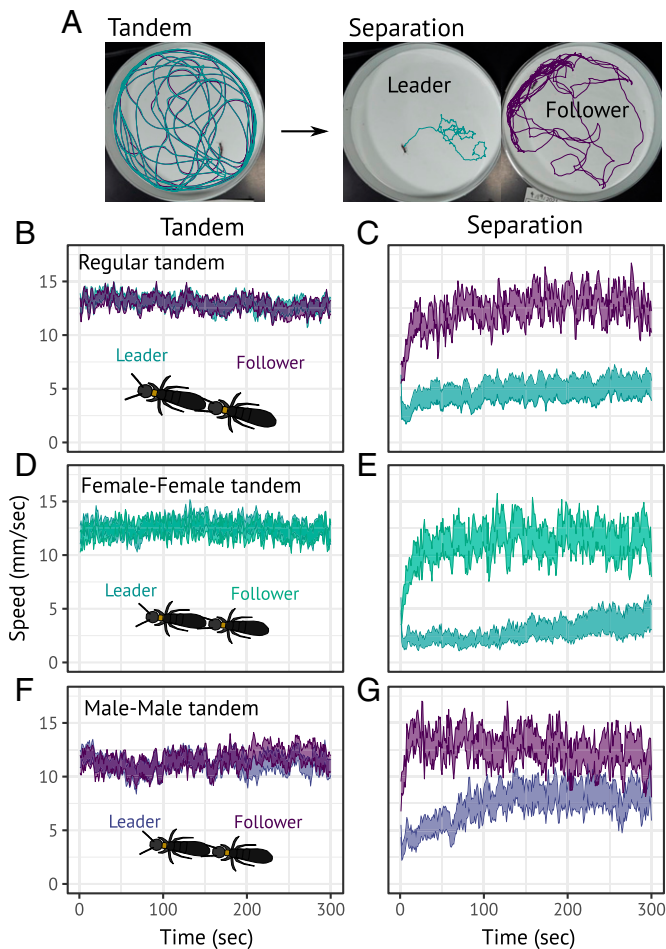


Fig. 1. Movement speed of termites in heterosexual or same-sex tandem pairings. (A) Representative 5-min trajectories during tandem run and after separation in heterosexual pairs. (B–G) Mean movement speed of individuals during tandems (B, D, and F) and after separation (C, E, and G). One individual was removed from each tandem at time = 0 in the right column to observe behavior after separation. Shaded regions indicate mean \pm 1 SE.

(SI Appendix, Fig. S4B). Also, leaders and followers showed distinct turning patterns after separation irrespective of pairing combinations, with leaders showing higher movement sinuosity than followers after separation (LMM, female–male: $\chi^2_1 = 7.137$, $P = 0.008$, effect size [d] = 0.734; female–female: $\chi^2_1 = 21.367$, $P < 0.001$, effect size [d] = 1.394; male–male: $\chi^2_1 = 8.988$, $P = 0.003$, effect size [d] = 0.816).

Dimorphic Movement Enhances the Likelihood of Tandem Reunion in Separated Same-Sex Pairs. In heterosexual tandem runs, sexually dimorphic movements after separation are key to efficient reencounters (18). We used an individual-based simulation model to examine whether the dimorphic movements observed in same-sex pairs are likely to enhance the probability of reunion after separation. In a random search, different movements result in different encounter rates (27). If male-behaving females or female-behaving males increase the likelihood of reunion in same-sex pairs, then dimorphic movements observed in same-sex pairs are predicted to increase search efficiency, compared to scenarios where both females paused or both males continued moving. Using parameters for speed and turning angles estimated from the analyses above, we modeled movement patterns using a correlated random walk (CRW). Then, we evaluated five combinations of movement patterns (Fig. 2A): the observed dimorphic movements in heterosexual

pairs, female–female pairs, and male–male pairs, and “virtual” monomorphic movement scenarios where both partners moved like female leaders or male followers.

Our simulations indicated that behavioral dimorphism in same-sex tandems increases reunion probability upon separation. In simulated male–male and female–female pairs showing dimorphic movements, the reencounter probability was comparable to that of heterosexual pairs, differing by less than 1% on average after 60 s (Fig. 2C). In contrast, if both males of same-sex pairs kept their behavior unchanged (i.e., both males moved to search for the stray partner), reencounter probability decreased by 5.26% on average after 60 s (Fig. 2C). The same was true for female–female pairs (Fig. 2C), for which reencounter probability decreased by an average of 12.33% after 60 s when both females kept their behavior unchanged (i.e., both females paused to wait for the stray partner).

Furthermore, we tested whether simulations that included plastic behavioral change reproduced empirical observations with reencounter rates better than those without such behavioral plasticity, by comparing simulated vs. empirical distributions of the time required for reencounter after separation (SI Appendix, Fig. S6). Similar reencounter rates were observed in simulations that incorporated sex-role plasticity (SI Appendix, Fig. S6 D–F), but not for simulations lacking such plasticity (SI Appendix, Fig. S6 G and H). The superior predictive power of simulations incorporating plasticity was consistent for male–male pairings (comparison of Kolmogorov–Smirnov [KS] D statistic, Wilcoxon rank sum test, $V = 0$, $P < 0.001$) (SI Appendix, Fig. S6J), and for female–female pairings (comparison of KS D statistic, Wilcoxon rank sum test, $V = 0$, $P < 0.001$) (SI Appendix, Fig. S6J).

Plasticity of Dimorphic Behaviors Required for Adaptive Same-Sex Tandems Is Ancestral. In *R. speratus*, both females and males possess a full behavioral repertoire for tandem pairing and can fully express behaviors associated with the other sex (Fig. 1). Previous studies have implied that sex roles may be flexible in early termite lineages (28), and we hypothesized that sex role flexibility in *R. speratus* was inherited from the common ancestor of modern termites. To test this hypothesis, we performed phylogenetic comparative analysis of termite tandem running behavior. We compiled information about termite tandem runs from 69 species of 35 genera by performing a systematic search of the literature (Materials and Methods and SI Appendix, Text S2, Fig. S9, and Tables S4 and S5) and including additional observations from three species representing two genera (SI Appendix, Text S5). Then, we mapped traits onto a genus-level phylogeny inferred from molecular and morphological data (29) and performed ancestral-state reconstructions to infer the tandem running behavior of the common ancestor of termites.

The focal species of this study, *R. speratus*, shows female-leader tandem runs in heterosexual pairs. All other taxa for which we found records in the Neoisoptera, the clade containing *R. speratus*, also show female-leader tandem runs ($n = 21$ genera) (Fig. 3). Our ancestral-state reconstruction indicated a 99.85% probability that tandem runs were present in the ancestor of Neoisoptera, and a 99.99% probability that those tandem runs were female-led. A separate reconstruction for males found a negligible probability (3.05%) of male-led tandems in the ancestor of Neoisoptera (Fig. 3).

In notable contrast, sex roles are more flexible in basal termite lineages, and tandem runs may be led by either females or males (Fig. 3). We found that the presence of tandem runs is likely ancestral in termites (95.47% probability). However,

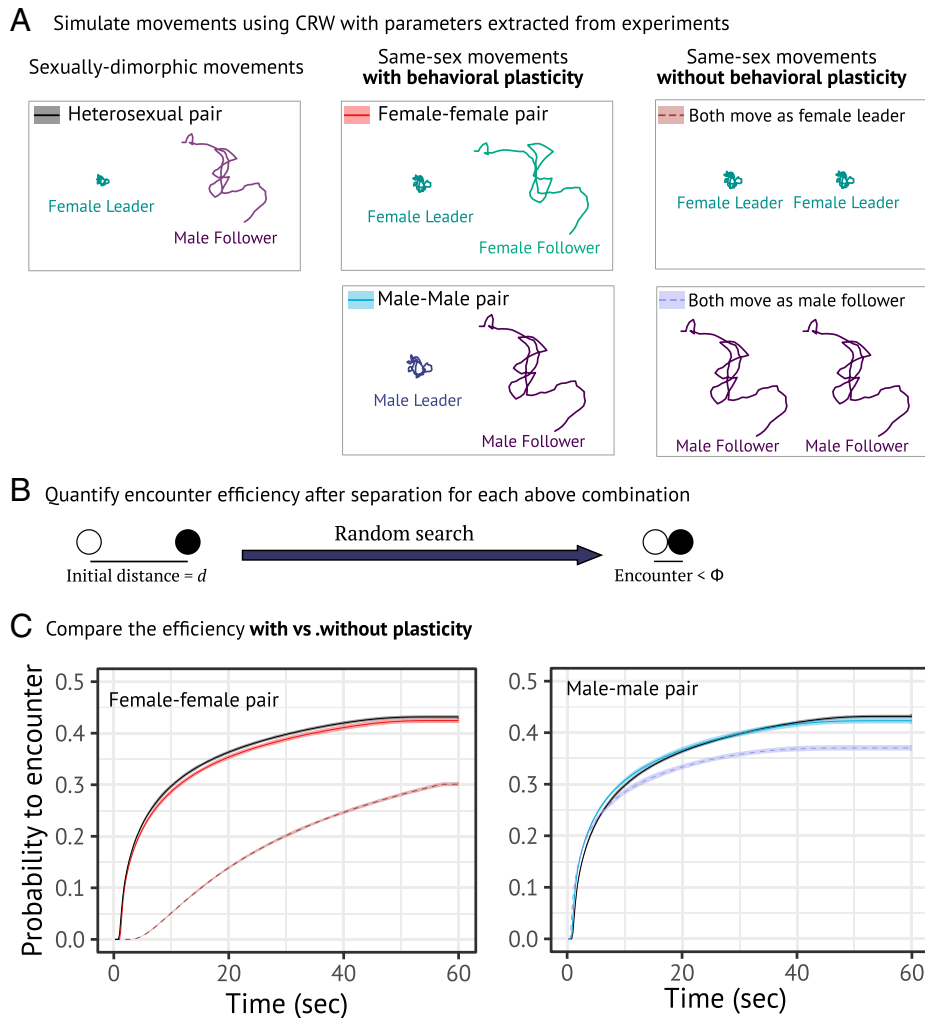


Fig. 2. Simulation results for reencounter efficiency of termite movements after pair separation. (A) Movement patterns for which we examined the searching efficiency. We extracted the movement patterns after separation for each sex and context. Using these movement patterns, we created five different combinations to measure the reencounter efficiency using individual-based model simulations. Movements were modeled using CRWs with parameters in *SI Appendix, Table S1*. Representative trajectories were produced using the same 100 random numbers for each parameter. (B) Simulation procedure. In the beginning, partners were separated by distance d ($= 16.766$), and we considered that they encountered when the distance became $< \Phi$ ($= 7$). (C) Comparison of encounter efficiency for movement combinations. In cases where one male in a male-male tandem expresses female-like movements upon separation, the resulting movement dimorphism enhances reencounter rates. Similarly, when one female in a female-female tandem expresses male-like movement, the resulting movement dimorphism enhances encounter rates. The probabilities to encounter were obtained by counting the number of pairs encountered over time among 100,000 simulations. By repeating 100,000 simulations 10 times, we obtained a mean ± 3 SD (shaded regions).

there was a large probability that both females and males expressed leader roles in the ancestor of modern termites (99.90% for females, and 79.95% for males) (Fig. 3). These results were based on an analysis using an outgroup lacking tandem runs; the probability that both sexes expressed leader roles in the ancestor of modern termites was higher when using an outgroup having tandem runs that were led by either sex (>94% for all traits) (*SI Appendix, Fig. S8*). Thus, before females acquired a fixed leader role in the heterosexual tandem runs of Neoisopteran termites, the leader-follower role was flexible. Both females and males were capable of expressing the full behavioral repertoire for movement coordination.

Discussion

Efforts to identify factors promoting the evolution of SSB have recently focused on the role of incomplete sex discrimination (5, 8), particularly in arthropod taxa (16, 30). Our results highlight a potentially important alternative that has received less attention: sex-role plasticity. We used the termite *R. speratus* to

experimentally determine that plasticity of sexually dimorphic behavior during tandem running is necessary for successful same-sex pair coordination in both sexes. In heterosexual tandems, leader females pause if they are separated from males, while follower males engage in an intensive search for their partner upon separation (Fig. 1 A–C). We found that both females and males retain the behavioral potential to flexibly adopt the role of the other sex when they interact in same-sex tandems (Fig. 1 D–G). Simulations parameterized with experimental data showed that sensitivity to sociosexual context and role flexibility are necessary for maintaining efficient same-sex tandems (Fig. 2), and comparative phylogenetic analysis indicated sex-role plasticity is ancestral in termites. Our results suggest that ancestral behavioral plasticity played an essential role in the evolution of SSB in termites. The importance of sex-role plasticity in SSB is likely to be widely relevant because expression of behaviors associated with the other sex is frequently observed in other taxa (9, 10). Therefore, our results have implications for understanding the nearly ubiquitous evolutionary maintenance of SSB across sexually reproducing animal taxa (1–4).

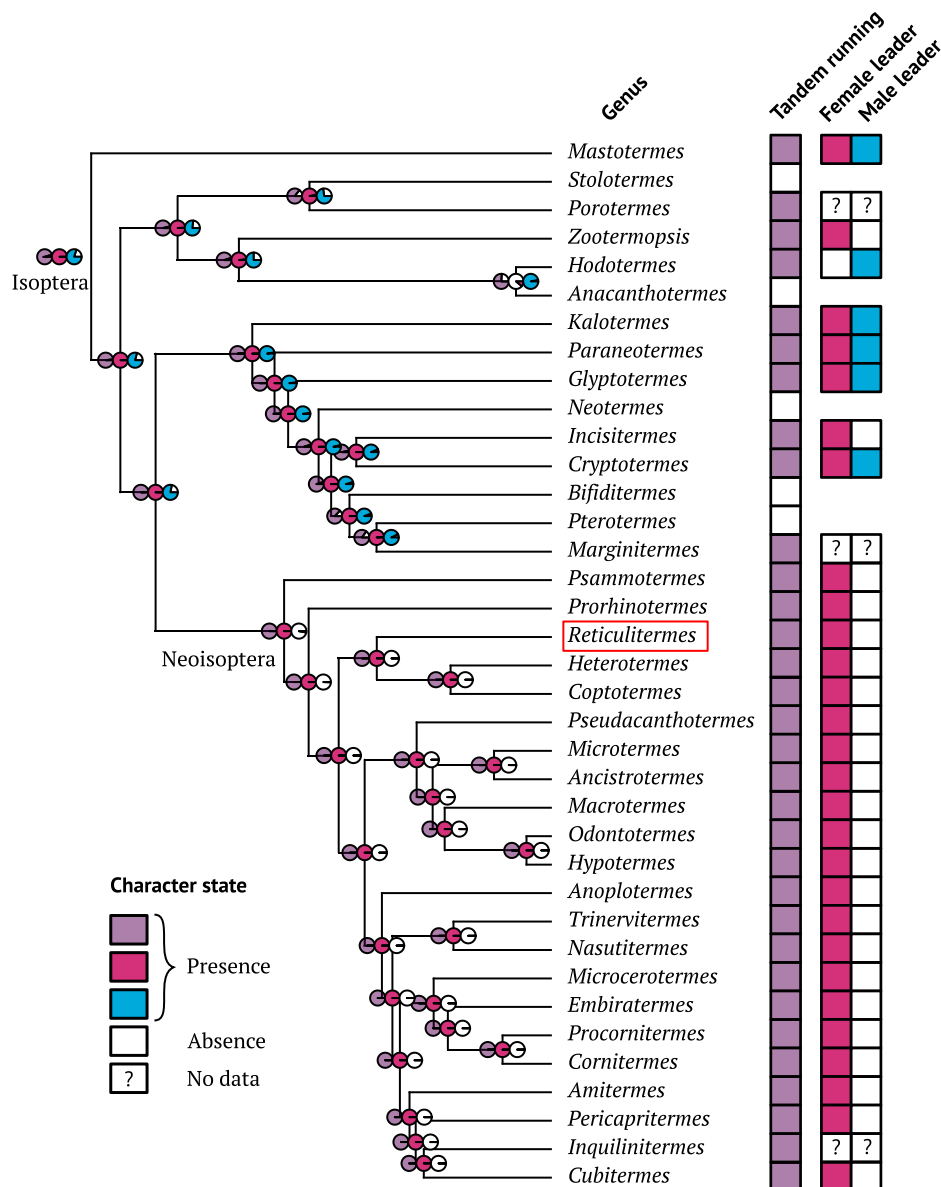


Fig. 3. Phylogeny of termites showing tandem run and leader role character states. The phylogenetic tree was simplified from Mizumoto and Bourguignon (29) and originally inferred from complete mitochondrial genome sequences and morphological characters. Pie charts at each node represent ancestral-state probability inferred from a maximum-likelihood model with equal trait change rates. Columns of shaded squares on the right indicate ancestral states inferred for tandem running (first single column), and female leader and male leader separately (second pair of columns). As an outgroup, *Cryptocercus* (not shown in the figure) was included in the ancestral-state reconstruction with a character of absence of tandem run. Results obtained from coding *Cryptocercus* with the presence of tandem running with both female and male leaders are shown in *SI Appendix, Fig. S8*. The genus *Reticulitermes*, used in behavioral observations during this study, is framed by a red rectangle.

In *R. speratus*, the behavioral plasticity that enables same-sex pairing relies on accurate sex detection and response to the immediate sociosexual environment. This finding contrasts with suggestions that the loss of sexual signals facilitates the evolution of SSB by reducing the ability to perceive and differentiate sexual signals, leading to “mistaken identity” (5, 30, 31). Even in cases where sexual behavior appears to be indiscriminate, it may be challenging to disentangle whether sex recognition does not occur, or whether it does occur but a decision is made to interact with a same-sex partner. In contrast, accurate sex recognition has been widely documented across sexually reproducing taxa [e.g., insects (32), reptiles (33), birds (34), amphibians (35), mammals (36)]. In neoisopteran termites, including *R. speratus*, only females have strong sex-pairing pheromones. In contrast, there is no such sex difference in basal lineages, with both sexes emitting pairing pheromones in some species and both sexes lacking them

in others (14, 37). Our results, therefore, emphasize that the assumptions of recent models for the evolution of SSB via indiscriminate mate choice (8) are not always met: same-sex pairing in termites occurs even with strong sex discrimination. Therefore, proximate mechanisms of SSB do not always require a lack of sex discrimination, even if it is expressed in an identical fashion between different sexes. Instead, we provide another plausible explanation that behavioral plasticity coupled with accurate sex discrimination underlies SSB. Future research would benefit from examining this possibility in a wider range of species, for example, using neuroethological methods to differentiate hormonal or gene-expression signatures of misdirected vs. intentional sexual interaction with same-sex conspecifics (11, 38).

A comparison between termite same-sex pairing and SSB associated with other mating systems provides useful insight into the evolution of mating behavior diversity. In same-sex

termite pairs, at least one individual expresses behavior associated with the other sex in the context of heterosexual pairing. Such expression of the other sex's behavior is known to evolve as an alternative mating tactic in many taxa. For example, in many insects, birds, and reptiles, males mimic females to avoid intraspecific competition (39, 40), prevent others from copulating with females (41, 42), or steal nuptial gifts (9). Similarly, females may mimic males to reduce the cost of copulation or male harassment (43). Such sexual mimicry might coevolve with SSB because SSB can also reduce aggressive interactions during competition (44, 45; but see ref. 46). However, same-sex tandems in termites are not likely to be directly comparable to alternative mating strategies such as those based on deception, because heterosexual pairing is the highest fitness option for all termites and represents a cooperative relationship. In contrast, alternative mating strategies can be the highest fitness option for some individuals depending on context, condition, or genotype, and the strategy is driven by competition dynamics (39).

Although SSB is frequently observed across species, its patterns of expression are highly diverse (47–49). In some species, only one sex expresses SSB, and in others, both sexes engage in SSB. The frequency and manifestation of SSB can also vary between sexes. Characterizing the sources of these differences can help clarify factors driving taxonomic diversity of SSB, and our results in *R. speratus* provide an illustrative case. Males were more likely to form same-sex tandems and compete to obtain follower positions, whereas females in same-sex tandems showed no such competition. To initiate tandem runs, at least one individual needs to begin following another individual. As females are the leaders in heterosexual tandems, one female must switch her behavioral strategy before forming a same-sex tandem. In contrast, males in heterosexual tandems adopt follower roles, meaning males can initiate same-sex tandems before they establish who takes the leader position. This asymmetry explains why females took longer to initiate same-sex tandems than males. Second, after separation, leader males quickly stopped waiting for the stray partner and moved away to search for another potential mate, while leader females showed longer pausing behavior (Fig. 2). This difference could be explained by the pair-bonding pheromones females secrete to attract males from a short distance (14). In a random search with sexual attraction signals, the signaling sex achieves higher encounter rates by moving slower or even pausing, while the sex without signals should move actively to enhance encounters (50, 51). Thus, pausing is not suitable for male termites if they cannot reencounter the stray partner in a short period, while for females, pausing can be beneficial for reencounters or encounters with new potential mates.

Recent theoretical work has proposed that SSB can be evolutionarily maintained through selection for indiscriminate sexual behavior (5), and a conceptual model has argued that the prevalence of SSB across widespread animal taxa might be due to inheritance of incomplete sex discrimination from a common ancestor of sexually reproducing species (8). However, these explanations for the prevalence of SSB in animals are based only on the viewpoint of active mates (i.e., interacting partners that attempt to court, mount, pair, or copulate with one another). The perspective of passive mates (i.e., interacting partners being courted, mounted, paired, or receiving a copulation attempt) is often lacking, and as a result, it is underappreciated that both individuals may modify their behavior during SSB to maintain same-sex pairing (10). In this study, we empirically demonstrated that one of the partners in a same-sex pairing of termites flexibly expresses the behavior of the other sex

to contribute to pair coordination. Importantly, accurate sex discrimination, rather than failures of it, and induction of plastic responses to the sociosexual environment, are both prerequisites for the stable occurrence of SSB. Therefore, it is important to expand our focus from incomplete sex discrimination to the diversity of factors that facilitate the evolutionary origins of SSB, particularly in taxa where sexually dimorphic interactions are involved.

Materials and Methods

Behavioral Observations. We collected *R. speratus* alates with a piece of nesting wood from five colonies in Kagoshima (colonies A and B), Miyazaki (colony C), and Fukui (colonies E and F) in March 2021, just before the swarming season. Each colony contained hundreds to thousands of alates. To control flight timing, all nesting wood pieces were maintained at 22 °C until experiments began. 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.

To ensure that we observed pair coordination for each combination equally, we first prepared a source group for tandem runs and then extracted a pair for further observations. Three source groups were used to generate heterosexual, female–female, and male–male tandems, respectively, consisting of: 1) 5 females and 5 males, 2) 10 females, and 3) 10 males. In each case, the 10 individuals were placed in a Petri dish ($\phi = 140$ mm) with moistened plaster. All individuals were marked with one colored dot of paint (PX-20; Mitsubishi) on the abdomen to distinguish individual identity. Groups were maintained for more than 30 min to ensure tandem formation. Each group was recorded with a video camera (HC-X1500-K, Panasonic), and we counted the number of tandem running individuals at 15 min. The number of tandem running individuals was compared across treatments using a GLMM with binomial distribution and logit link, where the “ID of the original colony” was treated as a random effect. A likelihood-ratio test was used to test for statistical significance of the inclusion of each explanatory variable (type-II test; here and for all relevant following statistical analyses). We ran a Tukey's post hoc test using the `glht()` function of the package “multcomp” and estimated effect sizes using the equation [10] in Nakagawa and Cuthill (52), with *z*-value or with *t*-value (here and for all relevant following statistical analyses). All members within the same source groups were from the same original colony. Note that tandem runs between nestmates frequently occur in *Reticulitermes* in natural conditions (e.g., 26.1% in *Reticulitermes flavipes*), and there is no nestmate avoidance in *R. speartus* during tandem formation (SI Appendix, Text S1). We used nestmates to provide a genetically consistent background across different treatments to attribute any observed behavioral change not to genetic differences but to behavioral plasticity. Our experimental procedures thus balanced rigorous experimental design requirements while minimizing biological artificiality.

We transferred a single tandem pair to an observation arena consisting of a Petri dish ($\phi = 90$ mm) with moistened plaster. We performed this transportation for one to three pairs simultaneously, depending on the availability of tandem pairs and video cameras. Individuals remaining in the original dish were not used for further experiments. Although the tandem was disturbed by transportation, most pairs restarted tandems after being introduced to the new arena. Once the tandem run resumed, we recorded the behavior of the pair for 5 min using the video camera described above. After 5 min, we carefully removed one individual using an aspirator and observed the movement of the remaining individual attempting reunion search (“separation search”). Throughout this process, termites experienced variable densities (from 10 individuals to an isolated pair or single individual). Note that this variation is not artificial but consistent with natural situations because the density of termite mate searchers can be highly variable; for example, the number of swarming alates was observed to range from the orders of 10 to 10,000 over days (53), and the number of potential mates progressively decreases due to predation and nest establishment within a limited pairing period (within a few hours) (13).

Each individual was used only once for data collection. We obtained 55 observations for heterosexual tandems (6, 13, 14, 8, and 14 for colonies A, B, C, E, and F, respectively), which included observations of separation search by 29 leader-females and 26 follower-males. Similarly, we observed 46 female–female

same-sex tandems (2, 10, 13, 8, 13), with 22 leader-female and 24 follower-female observations of separation search, and 56 male-male same-sex tandems (6, 12, 14, 8, 16), with 29 leader-male and 27 follower-male observations of separation search. We extracted the coordinates of termite movements from all videos using the video-tracking system UMATracker (54). We allocated "id 0" to the individual that was the leader at the beginning, and "id 1" for the follower, and manually checked all videos to verify that leader-follower roles were not inadvertently flipped during coordinate extraction. We down-sampled all videos to a rate of five frames per second (FPS) (= every 0.2002 s) for subsequent analyses. All data analyses were performed using R v4.0.1 (55).

Tandem Analysis. Our first analysis tested whether behavior differs in same-sex versus heterosexual tandems. To compare the time engaged in tandem runs across pairs, we automatically identified whether a pair was performing tandem runs for every video frame, combining methods described in previous studies (18, 56, 57). During observations, pairs were determined to be in one of three states: 1) tandem running, 2) interacting but not tandem running, and 3) searching (individuals in the pair are physically separated). We defined individuals in the pair as interacting (or tandem running) when the distance between their centroids was less than 7 mm (18). This distance slightly exceeds the average body length because termites in a tandem run are nearly in physical contact (18). An interacting pair was considered to be performing a tandem run only if they met the following criteria (56). First, the interaction needed to last for more than 2 s; a very short separation (<2 s) was not regarded as a separation event. Second, both termites needed to move more than 30 mm while interacting. After separation, we considered that individuals were engaging in separation search until they interacted again for more than 1 s. These thresholds (7 mm, 2 s, 30 mm) were determined based on previous studies (18, 56, 57). To assess the sensitivity of our analyses, we tested how modification of these thresholds affected the results (range: 6.5 to 7.5 mm, 1 to 3 s, 20 to 40 mm, respectively). These modifications slightly changed the proportion of the time spent in tandem runs, but the qualitative conclusion that same-sex tandems were not different from heterosexual tandems was consistent (SI Appendix, Fig. S2).

In male-male tandem runs, the two males occasionally chased each other, formed a small rotation, and competed over the follower position (Movie S1). We considered this competition state as a special case of tandem runs. The competition state was automatically defined as follows. First, the distance between individuals needed to be smaller than 4 mm as two individuals in such a state were located side by side in their heading direction and facing the opposite direction (Movie S1). Second, the rotation index of a pair needed to be larger than 0.5. The rotation index is calculated as the sum of the angular momenta about the center of the pair, taking values between 0 (no rotation) and 1 (strong rotation) (58). Finally, as in tandem runs, the above two conditions needed to persist for more than 2 s. These thresholds were determined according to visual inspection. As above, we confirmed that modification of these thresholds (range: 3.5 to 4.5 mm, 0.4 to 0.6, 1.5 to 2.5 s) did not change our conclusions (i.e., competition is observed in male-male pairs) (SI Appendix, Table S2).

We compared the proportion of time spent performing tandem runs across different pair combinations using an LMM, with pair combination (heterosexual, female-female, or male-male) treated as a fixed effect and the ID of the original colony as a random effect. We transformed proportional data using logit-transformation after adding 0.01 to the observed proportions to avoid infinite values (59). We also used a mixed-effects Cox model [coxme() function in the coxme package in R (60)], with pair combination treated as a fixed effect and the ID of the original colony as a random effect.

Movement Analysis upon Separation. In heterosexual tandems, a pair shows sexually dimorphic movements after separation in which leader females pause while follower males move to enhance chances of reunion (18, 56). To investigate how termites in same-sex tandems behave upon accidental separation events, we computed displacements of individual positions for every frame during tandem runs and after artificial separation. Then, we summed up five successive displacements to obtain moved distance per second as a proxy for movement speed (mm/s). We focused on the last 1 min for tandem runs and the first 1 min after separation to obtain the mean movement speed for each individual so that we could compare values across all combinations of sexes, roles, and contexts. The time of 1 min is based on a previous study that shows that sexual

dimorphism is most prominent during the first 1 min of 30 min after separation (18). Modifying this time window altered the results quantitatively (in the case of 30 s and 90 s, the estimated parameters varied within a range of 1.2 mm/s for speed and 0.04 for sinuosity from values reported in SI Appendix, Tables S1 and S3). These mean speeds were compared between leaders and followers for each pairing combination, using an LMM treating the leader-follower role as a fixed effect and the original colony as a random effect. Also, we compared speed among pairing combinations for leader and follower separately, with an LMM treating pairing combinations as a fixed effect and the ID of the original colony as a random effect.

In addition to movement speed, we analyzed sinuosity (turning patterns) of termite movements within the same time windows. We computed the turning angle as the magnitude of change in the direction of motion from one frame to the next frame. Then, we fit wrapped Cauchy distributions to turning angle data for each individual, using maximum-likelihood estimation methods, and took the distribution's scale parameter as a proxy for sinuosity (61). Depending on the value of the scale parameter, the wrapped Cauchy distribution varies from a uniform distribution (scale parameter = 0, maximum sinuosity Brownian walk) to a δ distribution (scale parameter = 1, minimum sinuosity straight walk). We compared the value of the scale parameter using an LMM as in the movement speed analysis. Although these parameters did not always follow normal distributions, LMM is robust against violations of distribution assumptions (62).

Individual-Based Model. We developed an individual-based model to test whether the observed behavioral dimorphism during separation search movement enhances reunion efficiency in same-sex pairs of termites to the same extent it does in heterosexual pairs. When termites in a pair are accidentally separated, the two individuals are close to each other, but uncertain where their partner is. This situation can be simulated by expressing the position of two random searchers located at distance d in a borderless two-dimensional continuous space (18) (Fig. 2A). The distance d was obtained from observed separated distances during spontaneous separation in the tandem observations above. We measured the mean separated distance for each separation event (345 events in total). The mean of these 345 mean separated distances was d ($= 16.766$ mm). Note that we did not consider encounters between a separated partner and a different individual because we focused on reunion efficiency. A previous study considered the effect of additional potential mates as a source of variation in the simulations (56). Our simulation considered that individuals search by random walk until reencountering a partner in a continuous space. An encounter was regarded to have occurred when the distance between the centers of the two individuals became smaller than ϕ . The value ϕ was based on our definition of tandem running ($=7$ mm) (Fig. 2A).

Individuals performed a CRW with parameters of speed and sinuosity, denoted v and ρ , respectively. The speed parameter v was obtained as the mean value of the movement speed for each sex and role during the 1 min after separation, while the sinuosity parameter ρ was obtained as the estimated scale parameter from the data of turning angles. Parameter values are summarized in SI Appendix, Table S1. Based on our behavioral analyses, each time step was adjusted to 0.2 s. Thus, each individual moved $0.2v$ mm in each time step. Turning angles followed wrapped Cauchy distribution with scale parameter ρ . After generating a uniform random number u ($0 < u \leq 1$), the turning angles θ were derived from the following equation by applying the inversion method (61):

$$\theta = 2 \arctan \left(\frac{1 - \rho}{1 + \rho} \tan(\pi(u - 0.5)) \right)$$

We initiated the simulation with a random bearing angle that fluctuated according to θ . At each step, the bearing angle was equal to the previous bearing angle plus the deviation θ such that the moving object always kept approximately the previous direction, forming a CRW. Our model is necessarily a simplification; movement models can be more complex, for example incorporating move/pause patterns (18), reorientation behavior based on Lévy walk (18), temporal changes of moving speed (56), and initial heading directions (56). Although including these complexities could affect the results quantitatively, previous studies show that movement speed is the most important parameter influencing dimorphic movements (18, 56).

We compared the searching efficiency among five combinations of movement patterns (Fig. 2A). These included: 1) female leaders in heterosexual pairs

and male followers in heterosexual pairs, 2) female leaders in same-sex pairs and female followers in same-sex pairs, 3) male leaders in same-sex pairs and male followers in same-sex pairs, 4) both female leaders in same-sex pairs, and 5) both male followers in same-sex pairs. Simulations were performed for 60 s (=300 time steps). We ran 100,000 simulations (= 100,000 different pairs) and measured efficiency as the number of pairs encountered over time (0.2 to 60 s). We repeated the 100,000 simulations for 10 iterations to examine the variability of the results. As a sensitivity analysis, we changed the parameters φ and $d \pm 10\%$ and 20% ($\varphi = 5.6, 6.3, 7.7, \text{ and } 8.4$; $d = 13.4128, 15.0894, 18.4426, \text{ and } 20.1192$) (SI Appendix, Fig. S7). Simulations were implemented in Microsoft Visual Studio C++ 2019.

If our simulations could capture the role of behavioral plasticity in the reencounter process, we expect that simulations accounting for behavioral changes should reproduce encounter dynamics empirically observed in termites better than simulations considering no behavioral changes. To test this idea, we examined the distributions of reencounter times for simulated pairs within 60-s time windows (SI Appendix, Fig. S6 A-H). Then, we compared the distributions obtained from simulations with distributions obtained from our empirical observations using the KS D statistic (SI Appendix, Fig. S6J). The D statistic is the maximum distance between two different distributions and is specifically used for model fitting of nonnormally distributed data (63). We obtained D statistics between empirical male-male pairs and simulations of male-male pairs or between empirical female-female pairs and simulations of female-female pairs for each iteration, then compared D statistics between simulations accounting for behavioral changes and considering no behavioral changes using Wilcoxon rank sum tests.

Comparative Analysis of Termite Tandem Running. To investigate the evolutionary process of termite tandem running behavior, we employed phylogenetic comparative analysis. We used genus-level information because we were interested in general evolutionary patterns found across termites. To obtain tandem running information from a variety of termite taxa, we conducted a systematic literature search following the recommendations for literature search described in the PRISMA statement for metaanalysis (64) (see SI Appendix, Fig. S9 for PRISMA flowchart). We examined 137 extant termite genera (SI Appendix, Table S4), which are included in the phylogeny used in Mizumoto and Bourguignon (29).

We conducted our literature search on 10th and 11th August 2022 using the online reference platform Google Scholar with the simple string function and search strings: "Genus-name" AND "tandem" (SI Appendix, Table S4). We used Google Scholar instead of other systems for metaanalysis, such as Web of Science or Scopus (65), because few studies have focused explicitly on termite tandem running behavior, and thus information on termite tandem runs usually does not appear in the title, abstract, and keywords (for example, the search result for the string, "tandem AND termite", recovered only 51 hits in Web of Science). The primary purpose of our systematic search was not to integrate results from multiple independent studies in the manner of a traditional metaanalysis, but to collect as much observational information on termite tandem runs as possible. Therefore, we included any observations from all sources, including original articles, reviews, books, proceedings, and doctoral theses, from the field of ecology, entomology, evolution, pest management, physiology, systematics, and taxonomy. In Google Scholar, we set the search range as "Any time," removed the "include citations" checkbox, and selected "Sort by relevance" and "Any type" (SI Appendix, Fig. S10).

We obtained 6,475 hits from Google Scholar searches in total, and the titles and provided abstracts from 4,323 records were screened (SI Appendix, Fig. S9 and Table S4). Then, we assessed the full texts of 156 records for eligibility (SI Appendix, Fig. S9 and Table S4). We also evaluated 28 reviews or books screened from 4,492 records (SI Appendix, Fig. S9). From the reference lists of these 184 records, we further added 16 articles to the full-text assessments, resulting in full-text assessments of 200 records (SI Appendix, Fig. S9). For records in non-English languages, we used DeepL Translator (<https://www.deepl.com/>) for assessments. We defined tandem running behavior as two individuals walking together in the form of one individual following behind the other individual (see SI Appendix, Text S2 for all screening and eligibility criteria in detail). As a result, we obtained information for 35 genera, including 69 species, from 59 records (SI Appendix, Table S5).

From all records, we identified whether tandem running behavior is present (1) or absent (0) for each species (SI Appendix, Table S5). If tandem running was

present, we also identified who performs the leader role (female, male, or both), whether tandem running behavior happens after shedding wings (1) or not (0) and, when information was available, whether same-sex tandem running occurred (SI Appendix, Table S5). Information was consistent across records within species as far as we examined (SI Appendix, Text S3). After assembling species-level observations, we integrated them into genus-level information. In all but one genus, information on tandem running behavior was consistent among species, and we pooled them together to obtain a genus representative code. The only exception was *Cryptotermes*, in which tandem was present in *Cryptotermes brevis*, *Cryptotermes cynocephalus*, and *Cryptotermes dudleyi*, but absent in *Cryptotermes domesticus* and *Cryptotermes havilandi* (SI Appendix, Table S5). However, the phylogenetic tree of *Cryptotermes* indicates that the two species lacking tandem running behavior are sister species nested within a group of species showing this behavior (66). Therefore, the phylogeny of *Cryptotermes* implies that tandem run was lost in the clade containing *C. domesticus* and *C. havilandi* and that tandem run is the ancestral condition in *Cryptotermes* (SI Appendix, Fig. S11). Because our interest in ancestral-state reconstruction was inferring the behavioral potential of each genus, we coded the tandem run as present with both sexes able to perform the leader role in *Cryptotermes* (SI Appendix, Table S5).

Some species lack clear evidence of tandem running behavior but show similar behavior in recorded observations. In *Mastotermes darwiniensis*, although there was no information about tandem runs by mating partners, workers perform tandem runs to recruit nestmates to new food resources (67). This indicates individuals of this species genetically possess the behavioral capacity to engage in tandem running, which we consider relevant to the context of our comparative analysis. Thus, we classified this species as performing tandem runs, with both females and males able to take the leader role because workers are not expected to show sexual behaviors. In *Hodotermes mossambicus*, mating pairs do not show the rigid pattern that is referred to as tandem running, but males emit pheromones to attract females, and females follow males when the pair moves (28). Generally, the presence of tandem running behavior is not entirely distinctive, where pairing is strong in Kalotermitidae, Rhinotermitidae, and Termitidae and weak in basal lineages like *Zootermopsis* (e.g., refs. 68 and 69). The following behavior observed in *H. mossambicus* was less clear than that seen in groups with strong pairing, but very similar to observations on *Zootermopsis angusticollis* (70). In addition, for the species that we identified as lacking tandem runs, it is explicitly noted that they lack following behaviors [e.g., forming a group aggregation instead of pairing (71), much weaker pairing than *Zootermopsis* (68), and no attraction or response to each other (72, 73)]. Thus, we regarded the following behavior of *H. mossambicus* as a variation of tandem running, and treated this species as tandem running with males expressing a leader role.

We also added tandem running information for *Heterotermes* and *Glyptotermes* based on our own behavioral observations (SI Appendix, Text S5). *Heterotermes* performs tandem runs with female leaders, based on the information of *Heterotermes aureus*. *Glyptotermes* performs tandem runs with either females or males as leaders, based on observations of *Glyptotermes fuscus* and *Glyptotermes satsumensis*. Furthermore, we also performed a systematic literature search for the tandem running information of the wood roach *Cryptocercus*, a sister group of termites, as an outgroup, using the string, "tandem" AND "Cryptocercus." We obtained 169 hits, screened 169 records, and assessed five full texts. However, we found no information on this genus. Therefore, we coded the tandem running behavior of this genus as either 1) no-tandem or 2) tandem with both leaders (which is consistent with the only observation of the tandem-like behavior in nontermite cockroaches) (74). We performed the analysis for both codings separately (SI Appendix, Fig. S8).

In summation, we compiled data from 37 genera, with 32 showing tandem running (23: female leader, 1: male leader, 5: both leader, and 3: without information of leaders) and 5 lacking tandem running. We performed ancestral-state reconstruction for tandem running using this information combined with the phylogeny reported in Mizumoto and Bourguignon (29). The phylogeny was inferred from previously published complete mitochondrial genome data combined with morphological characters. It consists of both extant and extinct species, where extant species were haphazardly selected so that one species represents one genus. Therefore, the species in the phylogeny do not necessarily match the species with tandem running information (see SI Appendix, Table S5 for how many genera have matched species). However, these two species are

more closely related to each other than to any other species used in the analysis. For the analysis, we pruned extinct species and extant species without available tandem information from the phylogenetic tree.

We carried out separate ancestral-state reconstructions for tandem running, female leadership, and male leadership, respectively, using the function `ace()` in the R package "phytools" (75). We used a maximum-likelihood model with an equal rate of transition among states.

Data Availability. All movement data and source codes for the analysis presented in this paper are available at Zenodo, DOI: [10.5281/zenodo.7040069](https://doi.org/10.5281/zenodo.7040069) (76).

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