

A Genomic Imprinting Model of Termite Caste Determination: Not Genetic but Epigenetic Inheritance Influences Offspring Caste Fate

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ABSTRACT: Eusocial insects exhibit the most striking example of phenotypic plasticity. There has been a long controversy over the factors determining caste development of individuals in social insects. Here we demonstrate that parental phenotypes influence the social status of offspring not through genetic inheritance but through genomic imprinting in termites. Our extensive field survey and genetic analysis of the termite *Reticulitermes speratus* show that its breeding system is inconsistent with a genetic caste determination model. We therefore developed a genomic imprinting model, in which queen- and king-specific epigenetic marks antagonistically influence sexual development of offspring. The model accounts for all known empirical data on caste differentiation of *R. speratus* and other related species. By conducting colony-founding experiments and additively incorporating relevant socio-environmental factors into our genomic imprinting model, we show the relative importance of genomic imprinting and environmental factors in caste determination. The idea of epigenetic inheritance of sexual phenotypes solves the puzzle of why parthenogenetically produced daughters carrying only maternal chromosomes exclusively develop into queens and why parental phenotypes (nymph- or worker-derived reproductives) strongly influence caste differentiation of offspring. According to our model, the worker caste is seen as a “neuter” caste whose sexual development is suppressed due to counterbalanced maternal and paternal imprinting and opens new avenues for understanding the evolution of caste systems in social insects.

Keywords: caste differentiation, social insect, genome imprinting, epigenetic inheritance, reproductive division of labor.

Introduction

Reproductive division of labor is a hallmark of social insects, where individuals follow different developmental trajectories resulting in distinct morphological castes (Wilson 1971). Increasing evidence in the last decade for heritable influences on division of labor has put an end to the assumption that social insect broods are fully totipotent and environmental factors alone determine caste (Smith et al. 2008; Schwander et al. 2010). The relative influence of environmental and heritable effects (genetic or epigenetic effects) on caste can vary among species along a continuum from strict environmental caste determination to almost pure genetic caste determination.

Termite colonies are typically founded by a monogamous pair of primary reproductives (one king and one queen) derived from alates (winged adults). In *Reticulitermes* termites, larvae differentiate into either the nymph-alate pathway (sexual pathway) or the worker pathway (neuter pathway; fig. 1). Most nymphs develop into alates and fly out to establish new colonies. Neotenic (also called secondary reproductives or supplementary reproductives) are produced from within the colony on the death of the primary king and/or queen and take over reproduction in the colony (Vargo and Husseneder 2009). Neotenic can develop either from nymphs to become “nymphoid” reproductives or from workers to become “ergatoid” reproductives (fig. 1). In the termite *Reticulitermes speratus* and other related species, the parental phenotypes (worker- or nymph-derived reproductives) influence the caste fate of the offspring (Hayashi et al. 2007; Kitade et al. 2010). These laboratory-based studies demonstrated that termite caste determination is not purely environmental but involves some heritable components. As a candidate mechanism by

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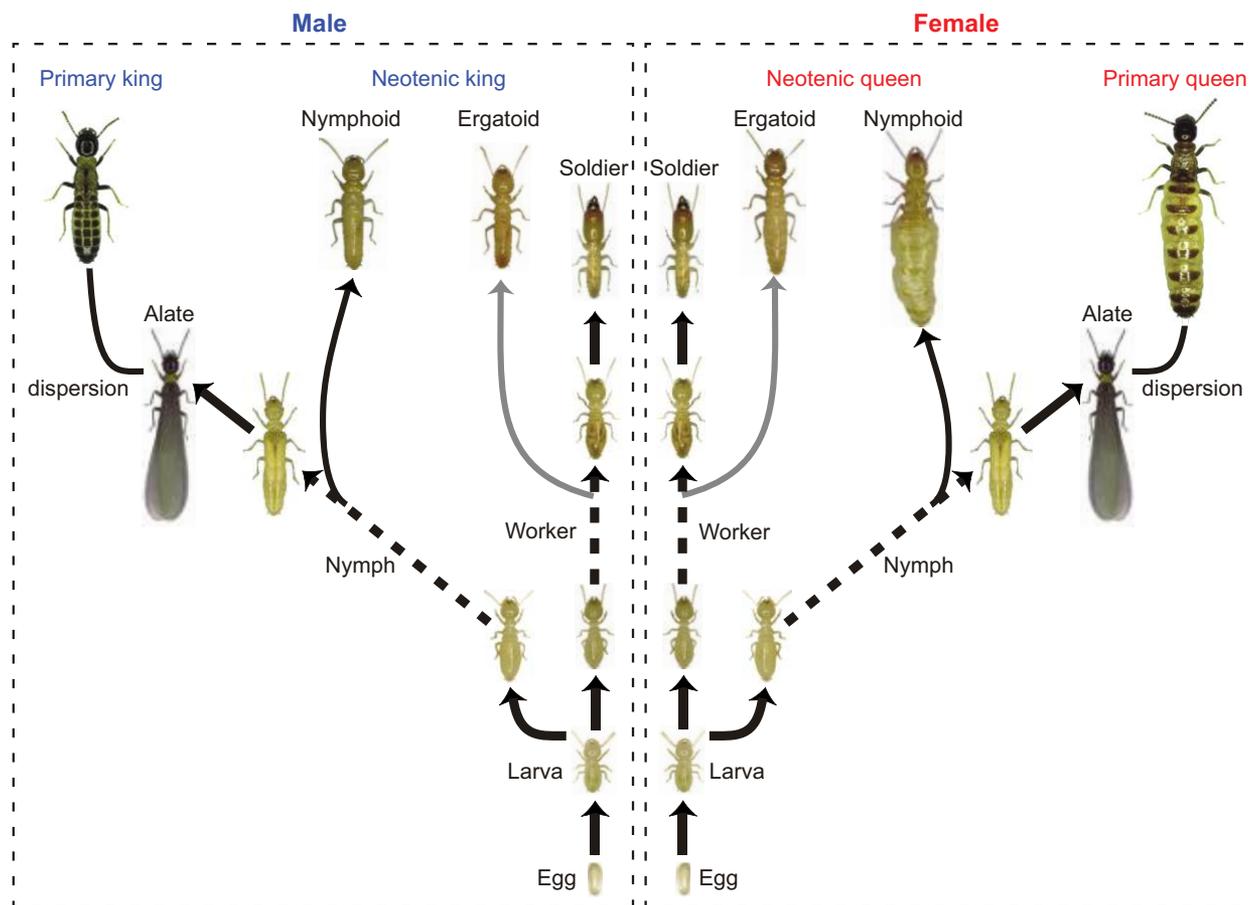


Figure 1: Caste differentiation pathway of *Reticulitermes* termites. Larvae differentiate into either the nymph-alate pathway (sexual pathway) or the worker pathway (neuter pathway). Nymphs develop into alates and fly out to establish new colonies. Nymphs and workers also have the potential to become nymphoids (nymph-derived neotenic reproductives) and ergatoids (worker-derived neotenic reproductives), respectively.

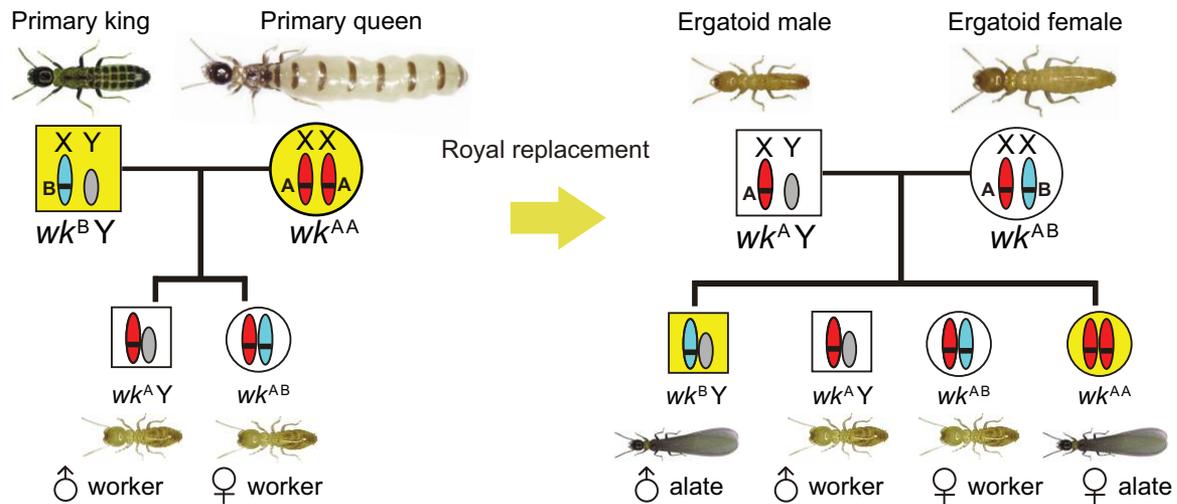
which parental phenotypes influence caste determination of offspring, Hayashi et al. (2007) proposed a genetically influenced caste determination (GCD) model (fig. S1; figs. S1–S5 are available online). The GCD model assumes the existence of an x-linked caste-determining locus *wk*; females have two x chromosomes and therefore three possible genotypes at *wk* (wk^{AA} , wk^{AB} , or wk^{BB}), and males have one x and one y chromosome (wk^AY or wk^BY ; fig. S1). Offspring with wk^{AB} and wk^AY genotypes develop into workers, and offspring with genotypes wk^{AA} and wk^BY develop into nymphs. The genotype wk^{BB} is lethal. The important prerequisite of this GCD model is that ergatoid (worker-derived) reproductives are very common and that breeding by ergatoid reproductives (wk^{AB} and/or wk^AY) is required for nymph production and therefore alate production (fig. 2a).

In contrast to the GCD model, by directly genotyping the royals (kings and queens) and other members of mature *R. speratus* colonies collected in the field, Matsuura et al. (2009) demonstrated that queens use parthenogenesis to produce nymphoid (nymph-derived) neotenic queens, which

supplement and then replace the primary queens. In this reproductive system, named asexual queen succession (AQS; fig. 2b), parthenogenetically produced individuals are destined to develop into neotenic and both the primary queens and the neotenic queens produce other colony members through sexual reproduction (Matsuura et al. 2009; Matsuura 2017).

It is apparent that AQS is contradictory to any genetic models including the GCD model proposed by Hayashi et al. (2007; fig. 2). Genetic models assume a genotype-phenotype linkage; that is, nymph-derived king and queen pairs (carrying nymph genotype[s]) produce worker genotype(s) (and thus worker phenotype). To maintain this genotype-phenotype linkage, the nymph-derived king and queen pairs must be replaced by worker-derived reproductives before producing nymphs. In the AQS system, however, both workers and nymphs are produced by the same combination of parental phenotypes—that is, nymph-derived kings and queens. Alate production by AQS colonies breaks the genotype-phenotype linkage, which is required for the maintenance of the GCD system (fig. 2). The discrepancy between genetic

a Hypothetical breeding system for the GCD system



b Asexual queen succession (AQS) system

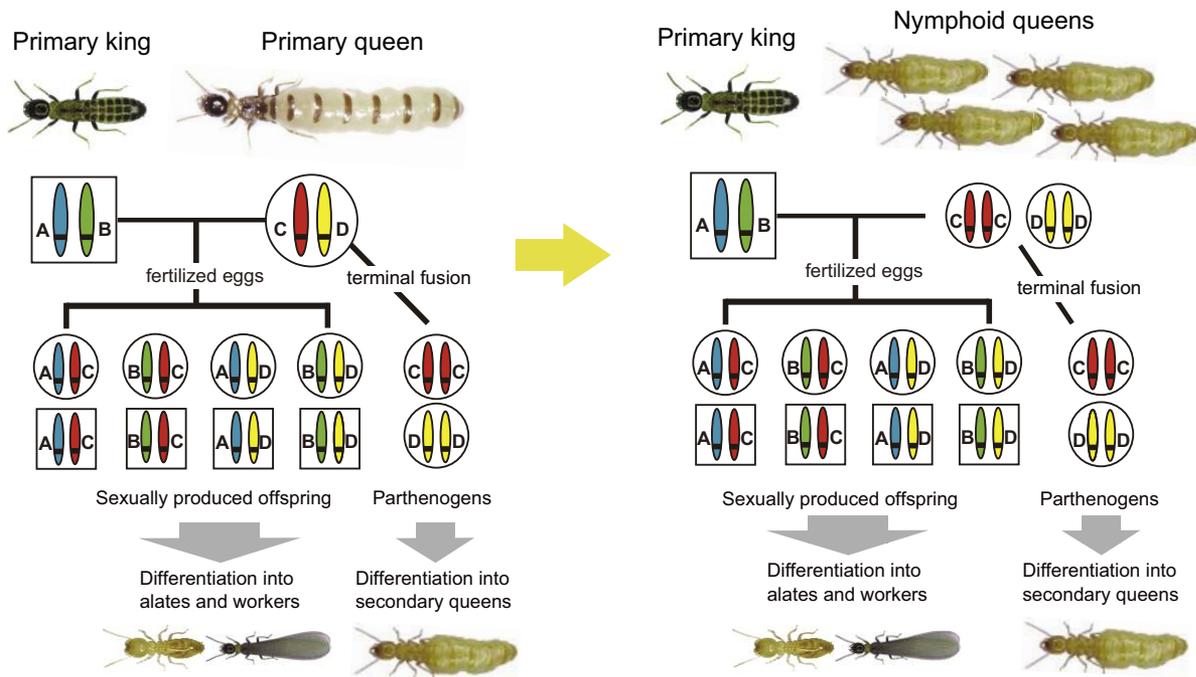


Figure 2: Contrasting the predictions of the genetically influenced caste determination (GCD) model and the asexual queen succession (AQS) system. *a*, The GCD model assumes a linkage between genotype and phenotype, so that offspring with wk^{AB} and wk^AY genotypes develop into workers and offspring with genotypes wk^{AA} and wk^{BY} develop into nymphs (and thus alates; fig. S1, available online). Primary reproductives must be replaced by ergatoid (worker-derived) reproductives (wk^{AB} and/or wk^AY) before nymph and alate production. *b*, AQS. Nymphoid (nymph-derived) queens produced asexually by the primary queen differentiate within the colony and replace the primary queen. Therefore, in the AQS system, both workers and nymphs are produced by the same combination of parental phenotypes, that is, nymph-derived kings and queens. Squares indicate males, and circles indicate females.

models and the AQS system can be explained by one of two possibilities: (1) there is geographic variation in AQS, where AQS can be found in a limited area and colonies of non-AQS populations have a GCD system; (2) the heritable influence of parental phenotypes is not due to genetic effects but results from other heritable factors.

In the latter case, a new epigenetic model can explain the heritable influence of parental phenotypes in termites that is consistent with AQS. Studies investigating heritable effects on caste determination might have largely overlooked epigenetic inheritance, although transmission to offspring of factors other than DNA sequences including epigenetic states can also affect offspring phenotype (Chong and Whitelaw 2004; Bonduriansky and Day 2008; Jablonka and Raz 2009). Recent studies of genomic imprinting show that genes can be differentially marked in egg and sperm and that inheritance of these epigenetic marks cause genes to be expressed in a parental origin-specific manner in the offspring (Sha 2008; Ferguson-Smith 2011). We here develop a genomic imprinting model, in which queen- and king-specific epigenetic marks influence caste determination of offspring, to understand the heritable influence of parental phenotypes and AQS in a single framework.

In this study, we first conducted wide geographic sampling of *R. speratus* colonies throughout its range in Japan and performed genotyping of royals and other members of each colony to identify kin structure. Genetic models predict that nymph-producing colonies have worker-derived reproductives, while our genomic imprinting model allows colonies with nymph-derived reproductives to produce both workers and nymphs. We found that *R. speratus* colonies are AQS throughout its range, where almost all nymph-producing colonies have only nymph-derived kings and queens. Therefore, instead of genetic models, we developed a genome imprinting model that accounts for all known laboratory experimental data on caste differentiation in *Reticulitermes* species as well as their reproductive systems in nature. We also examined caste development of offspring under the presence of founder males and females in incipient colonies to determine the relative importance of genomic imprinting and environmental factors in caste determination in *R. speratus*.

Material and Methods

Investigation of Field Colonies

Termite. The subterranean termite *Reticulitermes speratus*, which is widely distributed in Japan, is one of the most well-studied termite species with respect to its reproductive system (Matsuura and Nishida 2001; Matsuura et al. 2009; Kobayashi et al. 2013; Matsuura 2017), pheromone communication (Matsuura et al. 2007, 2010; Matsuura 2012; Mitaka et al. 2016), and caste differentiation (Miyata et al. 2004).

Like many other subterranean termites, *Reticulitermes* termites have cryptic nesting habits with transient, hidden royal chambers underground or deep inside wood, making the collection of reproductive individuals very difficult. We collected more than 2,000 nests in the field to obtain reproductives from a sufficient number of natural *R. speratus* colonies. We successfully found the royal chambers, where reproductives and young brood were protected, in 114 mature colonies collected in Honshu, Shikoku, Kyushu, Awajishima Island, Tsushima Island, and Yakushima Island, Japan, from 1998 to 2016. After finding young larvae and eggs, which indicate the presence of royal chambers nearby, we removed the parts of the wood containing the royal chambers using a chain saw and brought them into the laboratory for further dismantling. All reproductives were sampled by cutting the wood into ~15-cm-thick cross sections and carefully splitting the wood along the growth rings to expose termites inside. The reproductives from each colony were immediately preserved in 99.5% (vol/vol) ethanol with nestmate workers and nymphs in a vial. We distinguished primary reproductives (alate-derived) from secondary reproductives (neotenic) on the basis of a fully melanized body color and the presence of wing scales. Sex was determined from the configuration of the caudal sternites under a stereomicroscope (SZX7; Olympus). Neotenic reproductives were inspected for the presence or absence of wing pads to separate them into nymphoid (nymph-derived) and ergatoid (worker-derived) reproductives.

Microsatellite Analysis. We used 20 field colonies collected at Yakushima Island (colony code: YK150517A), Kagoshima, Kyushu (KG150730A), Kumamoto (SG150802C), Fukuoka (SH150801C), Tsushima Island (TS150730F), Ehime, Shikoku (UW150828C), Ehime (IM150829C), Kochi (KO150828A), Tokushima (MY150827B), Awajishima Island (MA150827I), Shimane, Honshu (MD150829D), Hiroshima (HI150829A), Okayama (TA090620A), Wakayama (GB130502C), Kyoto (UR060712A), Aichi (NG150711A), Nagano (MK160809A), Niigata (NI150720C), Ibaraki (MI100905I), and Aomori (MS150719E) for detailed genetic analysis (S2 data set; data sets S1, S2 are available online). We genotyped the single primary king, 10 randomly chosen secondary queens, and 20 randomly chosen workers (10 male and 10 female workers) from each colony. In colonies MI100905I and UR060712A, the primary queens were also present and genotyped. In colony MY150827B, only six secondary queens were collected, and all of them were genotyped (S1 data set shows the royal composition of each colony). Heads of individual termites were ground in Chelex 100 resin solution (Bio-Rad), and DNA was extracted and purified in accordance with standard Chelex-based protocols (Walsh et al. 1991). Individuals were genotyped at four highly polymorphic microsatellite loci: *Rf6-1*, *Rf21-1*, *Rf24-2* (Vargo 2000), and *Rs15* (Dronnet et al. 2004). Polymerase

chain reaction (PCR) conditions are detailed in previous studies (Vargo 2000; Dronnet et al. 2004); fluorescently labeled PCR products were analyzed in a 3500 Genetic Analyzer (Applied Biosystems) with the internal GeneScan-600 LIZ size standard (Applied Biosystems). Allele sizes were determined using GeneMapper 5 (Applied Biosystems). Secondary queens and workers were determined as sexual (containing both paternal and maternal alleles) or parthenogenetic (containing only maternal alleles) offspring based on the genotypes of the four microsatellite loci examined.

Phylogenetic Analysis. One worker randomly chosen from each of the 20 colonies used in microsatellite analysis was used for phylogenetic analysis. The sequences of other rhinotermitid species obtained in this study and from GenBank were also used in the analysis. Heads of individual termites were ground in Chelex 100 resin solution (Bio-Rad), and DNA was extracted and purified in accordance with standard Chelex-based protocols (Walsh et al. 1991). Individuals were sequenced for the mitochondrial COII gene. Primer sequences, PCR conditions, and sequencing methods are detailed in a previous study (Yashiro et al. 2011). Consensus sequences (658 bp) were aligned using the ClustalX algorithm from the BioEdit 7.0.4.1 sequence editor (Hall 1999) and were corrected manually. Bayesian inference was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with the GTR + G model selected by the hierarchical likelihood ratio test in MrModeltest 2.3 (Nylander 2004). *Heterotermes* sp. (GenBank accession no. KY302360) and *Coptotermes formosanus* (GenBank accession no. KM245826) were used as outgroups for the phylogenetic analysis. Two runs with four chains of Markov chain Monte Carlo iterations were performed for 2,000,000 generations, when the average standard deviations of split frequencies were below 0.01 (the first 25% of the generations as burn-in). Trees were kept for every 100 generations, and the latest 75% of the trees were used to calculate 50% majority-rule trees and to determine the posterior probabilities. The COII gene sequences obtained in this study were deposited in the DNA DataBank of Japan, European Molecular Biology Laboratory, and GenBank nucleotide sequence databases under the accession numbers KY302340–KY302360.

Genomic Imprinting Model

We developed a novel genomic imprinting model for caste predisposition in *R. speratus* and other *Reticulitermes* termites that closely matches empirical data from both lab and field studies. Sex-specific epigenetic marks (hereafter called “epimarks”) of the parents can influence the phenotype of the offspring through incomplete erasure of the epimarks in the germ line (Rice et al. 2016). Our genomic imprinting model assumes that heritable epimarks (i.e., imprinting) in-

crease in strength with the sexual maturation of the parents (fig. 3a). The sex- and phenotype-specific imprinting, which can influence the expression of both sex and growth regulatory genes, can be inherited, resulting in parent-specific gene expression in offspring (fig. 3b). The sex-specific imprinting effect of a parent of a certain phenotype on the development of offspring is given by

$$I_{\text{sex,phenotype}} = \delta_{\text{phenotype}}(s_{\text{sex}}, g_{\text{sex}}), \tag{1}$$

where sex is either male (m) or female (f) and phenotype is either ergatoid (E), nymphoid (N), or alate-derived primary reproductive (A). The values s_{sex} and g_{sex} are imprinting effects on the expression of sex regulatory genes and growth regulatory genes, respectively. The value $\delta_{\text{phenotype}}$ is the relative strength of imprinting effects of each parental phenotype. In the model, caste phenotype of the parents influences the imprinting level; nymphoid and primary reproductives, which develop from the reproductive pathway, obtain higher imprinting levels than ergatoids, which develop from the neuter pathway (figs. 1, 3a). Therefore, the values of $\delta_{\text{phenotype}}$ can be predicted to be $0 < \delta_E < \delta_N = \delta_A = 1$, although the range of δ_E is not theoretically constrained to be less than 1. The effects of maternal imprinting by a nymphoid queen $I_{f,N}$ and a primary queen $I_{f,A}$ on the development of offspring are given by

$$I_{f,N} = I_{f,A} = (s_f, g_f). \tag{2}$$

Because paternal imprinting acts antagonistically toward maternal imprinting with respect to the expression of sex regulatory genes, the effects of paternal imprinting by a nymphoid king $I_{m,N}$ and a primary king $I_{m,A}$ are

$$I_{m,N} = I_{m,A} = (-s_m, g_m). \tag{3}$$

The effects of imprinting by an ergatoid queen $I_{f,E}$ and an ergatoid king $I_{m,E}$ are

$$\begin{aligned} I_{f,E} &= \delta_E(s_f, g_f), \\ I_{m,E} &= \delta_E(-s_m, g_m). \end{aligned} \tag{4}$$

Let $s_m = ks_f$ and $g_m = kg_f$, where k is the relative strength of paternal imprinting to maternal imprinting, and let $g_f = rs_f$, where r is the relative strength of parental imprinting of growth factors to sexual factors. The effect of parental imprinting on the development of offspring is expressed as the sum of maternal and paternal imprinting. In the case of mating between a female nymphoid (fN) and a male nymphoid (mN), the effect of genomic imprinting I_{fNmN} is

$$\begin{aligned} I_{fNmN} &= I_{f,N} + I_{m,N} = (s_f, g_f) + (-s_m, g_m) \\ &= \{(1 - k)s_f, (1 + k)rs_f\} \end{aligned} \tag{5}$$

and that of mating between a female nymphoid (fN) and a male ergatoid (mE) is

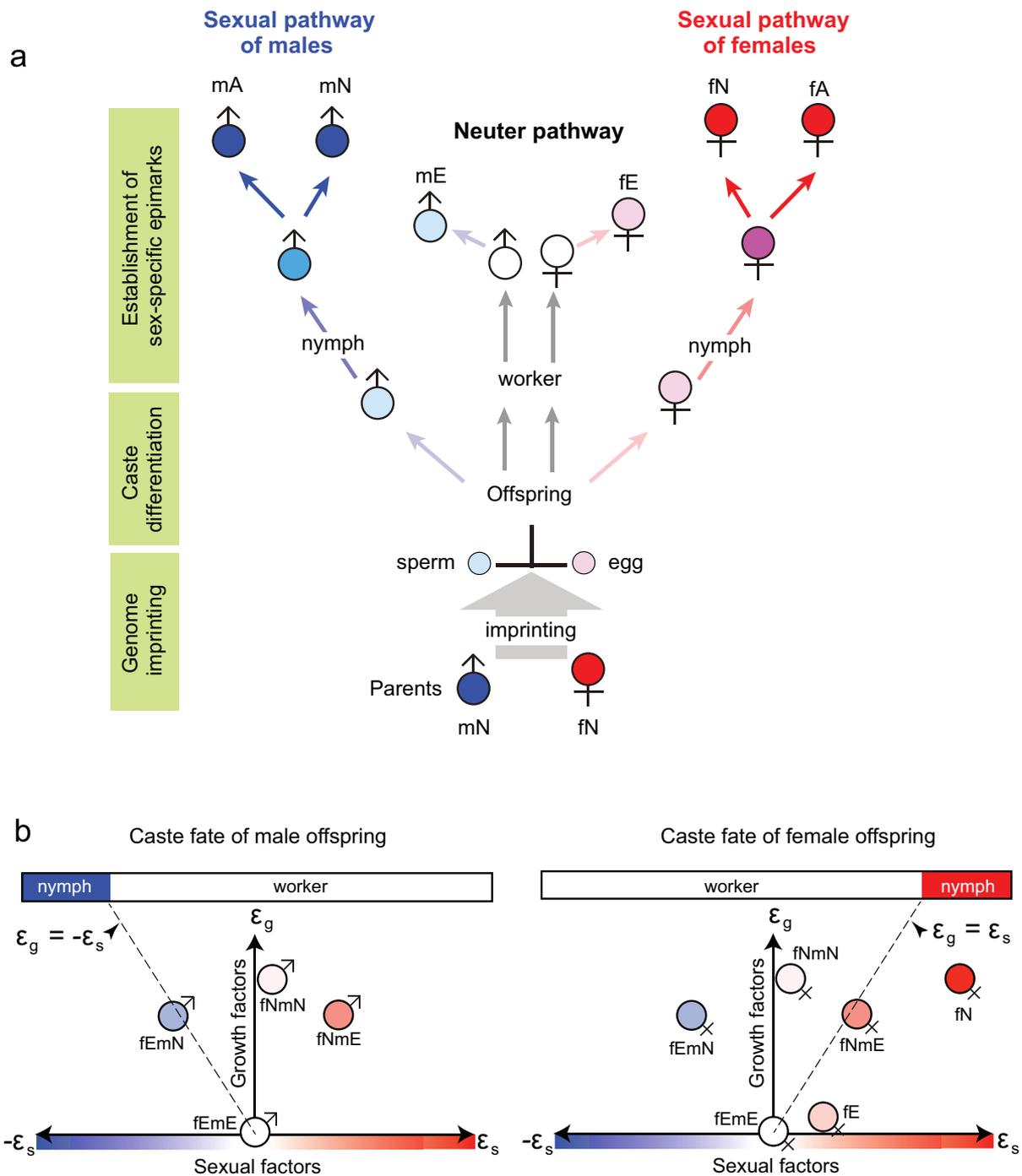


Figure 3: Scheme of our genomic imprinting model. *a*, Caste differentiation pathway and the establishment of sexual phenotype-specific imprinting. Males and females in the sexual pathway (nymph pathway) develop sex-specific epimarks. Incomplete erasure of sex-specific epimarks in the germ line results in genomic imprinting influencing gene expression in the offspring. Alates and nymphoids develop through five to seven molts of sexual development, while ergatoids develop by a single sexual molt (see also fig. 1). Blue and red arrows indicate the development of male- and female-specific epimarks, respectively. Darkness of the color represents the strength of epimarks. fA = female alate; fE = female ergatoid; fN = female nymphoid; mA = male alate; mE = male ergatoid; mN = male nymphoid. *b*, Expression levels of growth regulatory genes (growth factors: ϵ_g) and sexual regulatory genes (sexual factors: ϵ_s) of the male offspring (*left*) and female offspring (*right*). Male offspring with ϵ_g and ϵ_s values below the threshold line ($\epsilon_g = -\epsilon_s$) develop into nymphs. Female offspring with ϵ_g and ϵ_s below the threshold line ($\epsilon_g = \epsilon_s$) develop into nymphs. The size of each circle indicates developmental noise. Parents of the offspring were denoted by the following abbreviations: fE = female ergatoid (parthenogenesis); fEmE = female and male ergatoid; fNmN = female nymphoid and male nymphoid; fN = female nymphoid (parthenogenesis); fNmE = female nymphoid and male ergatoid; fNmN = female and male nymphoid. Primary reproductives fAmA have the same level of parental imprinting as fNmN.

$$\begin{aligned} \mathbf{I}_{fNmE} &= \mathbf{I}_{f,N} + \mathbf{I}_{m,E} = (s_f, g_f) + \delta_E(-s_m, g_m) \\ &= \{(1 - \delta_E k)s_f, (1 + \delta_E k)rs_f\}. \end{aligned} \tag{6}$$

In AQS species, in which females are capable of diploid thelytoky (parthenogenetic production of diploid female offspring), the effects of genomic imprinting on the parthenogenetic daughters produced by a female nymphoid \mathbf{I}_{fNFN} and a female ergatoid \mathbf{I}_{fEFE} are

$$\begin{aligned} \mathbf{I}_{fNFN} &= 2\mathbf{I}_{f,N} = 2(s_f, g_f) = 2(s_f, rs_f), \\ \mathbf{I}_{fEFE} &= 2\mathbf{I}_{f,E} = 2\delta_E(s_f, g_f) = 2\delta_E(s_f, rs_f). \end{aligned} \tag{7}$$

Caste fate of offspring is determined by the relative expression of growth regulatory genes (ϵ_g : growth factors) to sex regulatory genes (ϵ_s : sexual factors); growth without sexual development channels individuals to the neuter caste (i.e., workers), and growth with sexual development directs individuals to the reproductive caste (i.e., nymphs; fig 3b). This can be modeled such that offspring with ϵ_g and ϵ_s values below the threshold line (male: $\epsilon_g = -\epsilon_s$; female: $\epsilon_g = \epsilon_s$) develop into nymphs (fig. 3b).

Here, the effect of genomic imprinting on the sex and growth regulatory genes is denoted by $\mathbf{I} = (i_s, i_g)$. In the model, the developmental pathway of individuals is primarily determined by imprinting but also subjected to random variation due to developmental noise, that is, phenotypic variation among individuals with identical genotypes living in an identical environment (Scheiner et al. 1991). We incorporated the developmental noise into the model as follows: gene expression levels have uniform distribution within a circular region (radius = e) with center \mathbf{I} on the $\epsilon_s - \epsilon_g$ plane (fig. 3b). Thus, the proportion of female offspring with imprinting $\mathbf{I} = (i_s, i_g)$ destined to develop into nymphs is

$$\varphi_f[\mathbf{I}, e] = P\{\epsilon_g < \epsilon_s | (\epsilon_s - i_s)^2 + (\epsilon_g - i_g)^2 < e^2\}. \tag{8}$$

This can be calculated by

$$\varphi_f[\mathbf{I}, e] = \begin{cases} 0 & (d \geq e, i_g \geq i_s) \\ A_s/A_c & (d < e, i_g \geq i_s) \\ 1 - A_s/A_c & (d < e, i_g < i_s) \\ 1 & (\text{otherwise}) \end{cases}, \tag{9}$$

where d is the distance from the center \mathbf{I} to the threshold line ($\epsilon_g = \epsilon_s$), A_c is the area of the circle with center \mathbf{I} and radius e , and A_s is the area of the circular segment bounded by the circle and the threshold line.

Similarly, the proportion of male offspring with imprinting $\mathbf{I} = (i_s, i_g)$ destined to develop into nymphs is

$$\varphi_m[\mathbf{I}, e] = P\{\epsilon_g < -\epsilon_s | (\epsilon_s - i_s)^2 + (\epsilon_g - i_g)^2 < e^2\}, \tag{10}$$

and

$$\varphi_m[\mathbf{I}, e] = \begin{cases} 0 & (d \geq e, i_g \geq -i_s) \\ A_s/A_c & (d < e, i_g \geq -i_s) \\ 1 - A_s/A_c & (d < e, i_g < -i_s) \\ 1 & (\text{otherwise}) \end{cases}. \tag{11}$$

We can set e constant ($= 1$) since the magnitude of the noise is determined in relation to the strength of imprinting.

We tested whether our model can explain the observed caste differentiation patterns of offspring with various combinations of parental phenotypes (nymphoid or ergatoid) in *Reticulitermes* species (*R. speratus* [Hayashi et al. 2007], *Reticulitermes kanmonensis*, *Reticulitermes okinawanus*, and *Reticulitermes yaeyamanus* [Kitade et al. 2010]). We explored the parameter sets with the greatest explanatory power by calculating root mean square errors for the rate of nymphs produced by each combination of parental phenotypes. The parameter ranges are shown in table 1. We present a sensitivity analysis of the model, testing the effects of the s_f , δ , k , and r values on the caste fate of offspring (figs. S2–S5).

Table 1: Parameters used in our genomic imprinting model

Symbol	Definition	Range	Species			
			<i>R. spe</i>	<i>R. kan</i>	<i>R. yae</i>	<i>R. oki</i>
s_f	Maternal imprinting effects on sex regulatory genes	1.0–10.0	1.9	10.0	8.8	5.6
r	Relative strength of parental imprinting of growth factors to sexual factors	.01–1.00	.27	.88	.87	.98
k	Relative strength of paternal imprinting to maternal imprinting	.10–10.0	.85	.13	.19	.70
δ_E	Relative strength of imprinting effects of ergatoid to nymphoid	.01–1.00	.32	.02	.02	.09
RMSE151	.045	.049	.025

Note: We calculated the probabilities of individuals developing into nymphs across the range of parameters to explore parameter sets that can explain the empirical data of each species. The most explainable parameter sets are determined by using root mean square errors (RMSE) for the proportion of nymphs produced by each combination of parental phenotypes. Ranges and most fitted parameters are shown. *R. kan* = *Reticulitermes kanmonensis*; *R. oki* = *Reticulitermes okinawanus*; *R. spe* = *Reticulitermes speratus*; *R. yae* = *Reticulitermes yaeyamanus*.

During the colony-founding stage, the founder males and females strongly inhibit their first brood from differentiation into nymphs and encourage worker differentiation to obtain an initial labor force (Oster and Wilson 1978). We incorporated the effects of the founder males and females on offspring into our genomic imprinting model. The effects of queen presence $\varphi_{f,A}$ and king presence $\varphi_{m,A}$, likely through primer pheromones, are

$$\begin{aligned}\varphi_{f,A} &= (-s_p, g_p) = \eta(-s_f, rs_f), \\ \varphi_{m,A} &= (s_p, g_p) = \eta(s_f, rs_f).\end{aligned}\quad (12)$$

Here, we set the relative strength of s_p and g_p to s_f and g_f as η ($\eta = s_p/s_f = g_p/g_f, 0 < \eta < 1$). These effects on offspring are sex specific; that is, $\varphi_{f,A}$ acts only on female offspring and $\varphi_{m,A}$ acts only on male offspring. In a female-female (FF) colony, the effect of queen presence on female offspring is $2\varphi_{f,A}$. These effects are normally moderated after the emergence of first-brood workers. We predicted caste differentiation patterns with the effect of parent presence using the parameters determined for *R. speratus* in the above calculations. All calculations were performed using R, version 3.1.3 (<https://cran.r-project.org>).

Colony Foundation Experiment

In *R. speratus*, females that fail to pair with males can also found new colonies cooperatively with a female partner and produce offspring by parthenogenesis (Matsuura and Nishida 2001; Matsuura et al. 2002, 2004). To assess the relative effects of genomic imprinting and socio-environmental factors, we took advantage of the ability of female-female pairs to successfully found colonies. We investigated the parental effect on caste differentiation of offspring in incipient colonies by comparing caste compositions between 1- and 5-year-old colonies founded by female-female (FF) and male-female (MF) pairs. Three nests (A, B, and C) of *R. speratus* were collected in May 2001 in Kaigara-yama, Okayama, and Takaragaike and Kamigamo, Kyoto, western Japan, respectively. The nests were maintained in a plastic box (200 mm × 350 mm × 200 mm) covered with nylon mesh and were held at 15°C in the laboratory for 5 days to control the timing of flight. Just prior to the experiments, the box was transferred to a room at 30°C; soon after, alates emerged from the nest wood and began to fly. The alates were then separated by sex, determined using external abdominal characters. Alates were used for the experiment within 24 h of flight.

Dealated males and females were randomly chosen from each colony and assigned to FF or MF pairs. All combinations of the three colonies were used to create the FF and MF pairs, with 15 replicates for each combination. An additional 50 FF and 50 MF nestmate pairs were made using the adults of a colony (D) collected in Kyoto to supplement

replications at 5 years in the case of low survivorship. In total, we prepared 140 FF and 185 MF pairs. Each pair was placed in a petri dish (90 mm in diameter) with a mixed sawdust food block (40 mm × 40 mm × 10 mm) and kept in the dark at 25°C. The colonies were watered every 2 months. At 1 year, 24 FF and 32 MF colonies were randomly chosen from the surviving colonies of the combinations of A, B, and C original colonies. At 3 years, pine sawdust was added to each colony. At 5 years, 16 FF and 16 MF colonies were randomly chosen from the surviving colonies of the combinations of A, B, and C original colonies. Because the pairs including alates of the original colony A did not survive at 5 years, we additionally used 10 FF and 10 FM nestmate pairs of colony D for analysis. The nests were carefully dissected to extract the primary reproductives and their progeny, including eggs, larvae, workers, soldiers, nymphs, and supplementary reproductives. The proportion of nymphs among the offspring was compared between FF and MF colonies at 1 and 5 years using a χ^2 test. Eggs and larvae were excluded from the caste composition analysis because neuter and reproductive lines cannot be distinguished in the immature stages. Since nestmate and nonnestmate pairs showed no significant difference in any measurements, we pooled the data on colonies founded by nestmates and nonnestmates.

Results

Rejection of Genetic Influence Hypothesis

Our wide-area assessment showed that *Reticulitermes speratus* exhibits no geographic variation in its reproductive system (fig. 4a). We performed microsatellite analysis on the 20 representative colonies collected from throughout its range in Japan to determine parentage of the neotenic queens and other colony members. Our genetic data showed that all *R. speratus* colonies collected in all areas exhibited AQS; that is, neotenic queens are produced by parthenogenesis of primary queens (fig. 4a; S2 data set). Among these 20 colonies, all colonies had parthenogenetically produced secondary queens, where nearly all of the secondary queens (158 of 165) were produced by parthenogenesis (see the S2 data set for raw genotyping data). Therefore, it is clear that geographic variation cannot explain the inconsistency of the genetic model (Hayashi et al. 2007) and AQS (Matsuura et al. 2009).

We found that among the 162 kings collected from 114 mature colonies in the field, 104 were primary and 58 were nymphoid, while no ergatoid king was found in the field. Among the 6,824 queens, 6,812 (99.82%) were nymphoid and six were primary (alate-derived), while only six (0.088%) were ergatoid (fig. 4b; S1 data set). Moreover, all of these mature colonies had nymphs. These results are clearly inconsistent with the prediction of the genetic influence hypothesis that nymph-producing colonies have ergatoid reproductives.

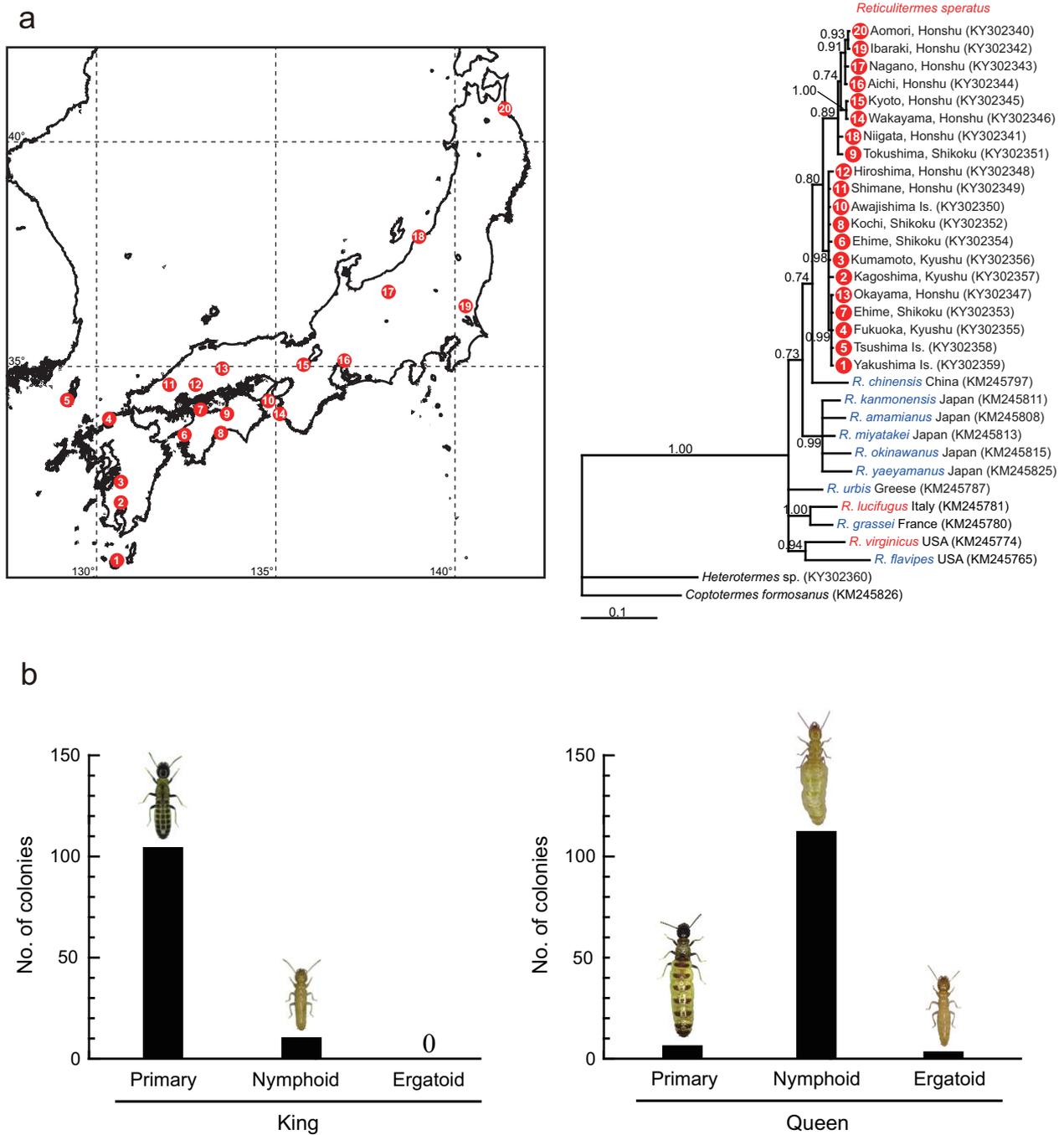


Figure 4: Wide-area sampling of mature colonies of *Reticulitermes speratus*. *a*, Geographic distribution of asexual queen succession (AQS) colonies (*left*) and a Bayesian phylogenetic tree (*right*) of the colonies (colony identification number 1–20) based on mitochondrial COII sequences. Red circles indicate AQS colonies. In the phylogenetic tree, AQS species are indicated in red and non-AQS are in blue. The corresponding posterior probabilities (≥ 0.70) are shown by the branch. The horizontal bar represents a distance of 0.1 substitutions per site. GenBank accession numbers are shown in parentheses. *b*, Sexual phenotypes of kings and queens collected from mature colonies producing alates.

For example, the GCD model (Hayashi et al. 2007) assumes a link between genotype and phenotype so that offspring with wk^{AB} and wk^{AY} genotypes develop into workers and offspring with genotypes wk^{AA} and wk^{BY} develop into nymphs (fig. S1). The important prerequisite of this GCD model is that primary reproductives must be replaced by ergatoid (worker-derived) reproductives (wk^{AB} and/or wk^{AY}) before nymph and alate production (fig. 2a). Our field data refuted this assumption as described above. Hence, the GCD model cannot explain the caste system of *R. speratus*. This requires an alternative model to explain why parental phenotypes (nymphoid or ergatoid) strongly influence the caste fate of offspring (Hayashi et al. 2007; Kitade et al. 2010).

Genomic Imprinting Model Results

Sensitivity analysis of our genomic imprinting model showed that each of the parameters (s_p , r , k , and δ_E) influences the caste fate of the offspring depending on the combination of parental phenotypes (figs. S2–S5) and found the parameter combinations with the greatest explanatory power for the empirical data (table 1). The prediction of our genomic imprinting model matched the empirical data for caste differentiation patterns in the AQS species *R. speratus* (Hayashi et al. 2007) and the non-AQS species *Reticulitermes kanmonensis*, *Reticulitermes yaeyamanus*, and *Reticulitermes okinawanus* (Kitade et al. 2010; fig. 5a) when the model parameters are allowed to vary among species (table 1). In addition, the GCD model does not explain the differentiation pattern of *R. okinawanus* (Kitade et al. 2010), whereas our genomic imprinting model successfully accounts for the observed pattern.

Effects of Socio-environmental Factors

In the 1-year-old FF colonies, 98.8% of the parthenogenetic daughters differentiated into workers (fig. 5b; table S1; tables S1–S4 are available online) in contrast to the results under orphaned conditions in older colonies (Hayashi et al. 2007), indicating that the imprinting effect on caste predisposition is smaller than the strong parental effects presumably involving pheromones at the early stage of colony founding. In the 5-year-old FF colonies, 9.0% of the parthenogenetic daughters differentiated into nymphs (7.4% nymphs and 1.6% nymphoid queens; table S2), which was significantly higher than in the 1-year-old FF colonies ($\chi^2 = 18.63$, $P < .0001$). The MF colonies had neither nymphs nor nymphoids even at 5 years (fig. 5b; table S4). The significant difference between 5-year-old FF and MF colonies in the proportion of nymphs ($\chi^2 = 152.7$, $P < .0001$) indicates that the effects of genomic imprinting in these older colonies were strong enough to overcome the inhibitory power of royal pheromones.

By additively incorporating the socio-environmental factors into our genomic imprinting model, we can explain the empirical data of caste differentiation in the presence of founding queens and kings (fig. 5c). Socio-environmental effects also include the effect of workers. During the transition from the ergonomic stage to the reproductive stage of colony growth (Oster and Wilson 1978), sufficient numbers of workers should suppress offspring from developing into workers and encourage nymph differentiation. This can explain why the offspring of a primary king and nymphoid queens are able to differentiate into alates in natural colonies. Critically, some parthenogenetic daughters differentiated into nymphoid queens in the presence of reining queens in our FF colonies, while no such reproductive differentiation occurred in the MF colonies (fig. 5b). This can be explained by our genomic imprinting model because parthenogenetic daughters are expected to have double the maternal imprinting effect, thereby strongly directing them toward sexual maturation rather than the sexually produced daughters who would have half-paternal and half-maternal imprinting. This demonstrates the adaptive function of genomic imprinting in the life cycle of AQS termites, where parthenogenetic daughters almost exclusively differentiate into neotenic queens.

Discussion

We conclude that our genomic imprinting model is a viable model to explain the influence of parental phenotypes on the caste predisposition of the offspring, while the assumption of genetic models was clearly rejected by our field data. In *Reticulitermes* species, both parental phenotype and environment affect caste differentiation patterns. To harmoniously incorporate these factors, our model assumes that the expression of sexual regulatory genes and growth regulatory genes in offspring are affected by heritable factors (i.e., parent sex- and phenotype-specific imprinting) and by socio-environmental factors (e.g., pheromones and nutritional condition). In our model, the relative expression of sexual and growth regulatory genes determines the developmental pathway of individuals. Males and females differentiated into either the worker pathway or the nymph pathway and establish their de novo phenotype-specific epimarks, which can be transgenerationally inherited by their offspring through eggs and sperm (fig. 3a). Therefore, our model can reasonably incorporate both heritable factors and environmental factors into the life cycle of these termites and matches the reproductive system in nature as well as the results of laboratory experiments.

The presumed evolutionary pathway leading to our genomic imprinting system is plausible and parsimonious. With the development of reproductive division of labor, sexual dimorphism of the reproductive castes should increase; that is, kings and queens should have greater differences in

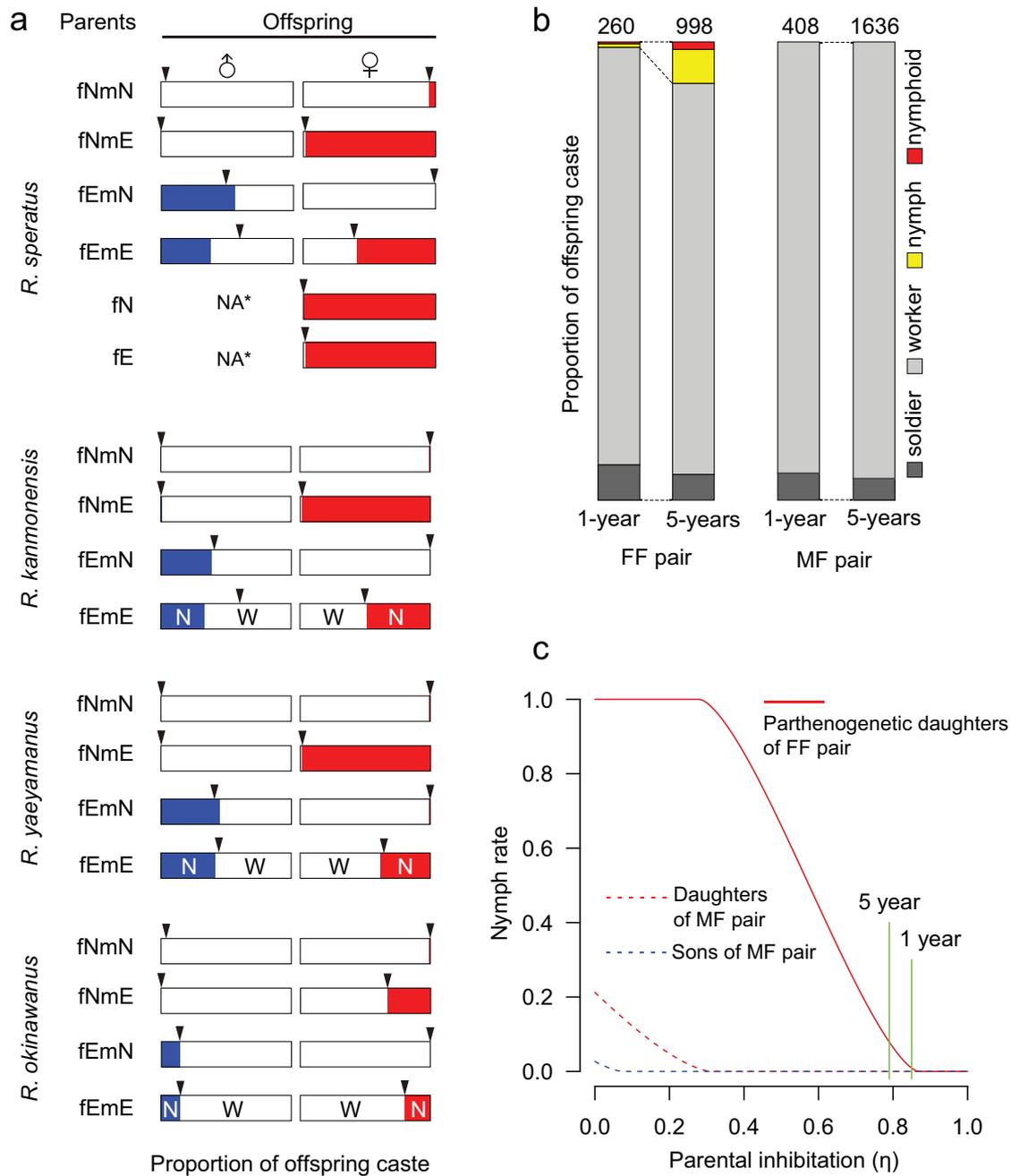


Figure 5: Comparison of our genomic imprinting model predictions and empirical data. *a*, The caste differentiation patterns predicted by our genomic imprinting model (bar graphs) and the empirical results (arrowheads) of *Reticulitermes speratus* (Hayashi et al. 2007), *Reticulitermes kanmonensis*, *Reticulitermes yaeyamanus*, and *Reticulitermes okinawanus* (Kitade et al. 2010) when model parameters are allowed to vary among species (parameter values are shown in table 1). Parthenogenesis produces only female offspring. *b*, Caste differentiation of offspring in the presence of parents. Proportion of offspring caste in incipient colonies founded by female-female (FF) and male-female (MF) pairs at 1 and 5 years of age. In FF colonies, founding females produce only female offspring by parthenogenesis. Nymph and nymphoid queens differentiated only in the FF colonies. *c*, Our genomic imprinting model prediction in the presence of parents. The model prediction matches well with the empirical results of caste differentiation in FF and MF colonies.

their sexual traits so as to be specialized in sperm and egg production, respectively. Development of sexual dimorphism requires sex-specific gene expression, and sexual dimorphism is often linked to the establishment of sex-specific epigenetic marks, which are involved in canalizing sexual development (D'Ávila et al. 2010; Ferguson-Smith 2011; Glastad et al. 2016). Sex-specific epimarks would contribute to king- and queen-specific gene expression and thus increase reproductive output. Incomplete erasure of these epimarks in the germ line would lead to transgenerational carryover of the sex-specific epimarks, which could act antagonistically in the offspring influencing their sexual development (Rice et al. 2016). It is unnecessary to invoke novel mechanisms for the evolution of our genomic imprinting system. Hence, our model matches well with the reproductive systems of a broad range of termite species.

Note that recent studies identified three AQS species in higher termites (Termitidae), which are phylogenetically distant from the lower termite genus *Reticulitermes* (Fougeyrollas et al. 2015, 2017; Fournier et al. 2016). The termitid species with AQS exhibit different mechanisms of parthenogenesis: *Cavitermes tuberosus* uses automixis with terminal fusion (Fournier et al. 2016), resulting in homozygous female parthenogens as in *Reticulitermes*, whereas *Embirtermes neotenicus* and *Silvestritermes minutus* use automixis with central fusion, resulting in heterozygous parthenogens (Fougeyrollas et al. 2015, 2017). Although there is no genetic difference between the founder queen, which is derived from an alate, and her parthenogenetic daughters in the latter species, the parthenogenetic daughters (carrying only maternal chromosomes) also have a higher propensity to develop into neotenic queens than sexually produced daughters (carrying paternal and maternal chromosomes). Thus, our genomic imprinting model also matches the situation in the higher termite species. In addition, only our genomic imprinting model can reasonably explain the evolution of AQS in termites. The origin of AQS requires the simultaneous evolution of two components: the capacity for parthenogenesis and the higher propensity of parthenogens to develop into neotenic queens than sexually produced daughters (Matsuura 2017). Our model provides a mechanism for the simultaneous evolution of parthenogenesis and the developmental caste bias of parthenogens.

The basic idea of our genomic imprinting model, that maternal and paternal imprinting act on the offspring's sexual development in different directions, is in accordance with the sexual antagonism theory of genomic imprinting (Day and Bonduriansky 2004; Rice et al. 2016). An example of the sexual antagonism of parental imprinting can be seen in the genomic imprinting sex determination mechanism in some haplodiploid insects (Dobson and Tanouye 1998; Gadagkar 2000). One novel prediction of genomic imprinting is that the neuter termite worker is generated by the counter-

balanced actions of maternal and paternal imprinting on the offspring's sexual development. Another leading evolutionary explanation for genomic imprinting is kinship theory, which predicts that differences in inclusive fitness between mothers and fathers can lead to conflicts between maternal and paternal genes in their offspring (Haig 2000; Queller 2003; Burt and Trivers 2006; Kronauer 2008; Drewell et al. 2012; Kocher et al. 2015; Galbraith et al. 2016). Our genomic imprinting model and the kinship theory of genomic imprinting are not mutually exclusive since the former deals with the mechanism of caste determination and the latter concerns evolutionary dynamics. However, it is important to note that our genomic imprinting model can explain the observed caste predisposition in termites without assuming any kin conflict between kings and queens.

Our genomic imprinting model provides qualitative predictions for future studies of the molecular mechanisms by which parental phenotypes influence the caste fate of offspring. Our prediction of the genomic imprinting parameters indicates that in all of the study species the strength of maternal imprinting should be greater than paternal imprinting ($k < 1$; table 1), suggesting that females have higher levels of epimarks (e.g., higher DNA methylation or histone modification). Similarly, nymphoids have stronger imprinting effects on the offspring than ergatoids ($\delta_e < 1$; table 1). In this article, we proposed a simplified model to explicitly demonstrate the influence of genome imprinting on caste determination. To obtain quantitative predictions for interspecific comparisons of parameter values, however, we would need to explore other factors that may affect caste differentiation. For instance, the effect of phenotypes on imprinting strength might differ between sexes; that is, δ can be different between males and females. The parameter r , the relative strength of parental imprinting of growth factors to sexual factors, can also be different between sexes or among phenotypes. In AQS species the original queen is replaced by multiple parthenogenetically produced daughter queens; that is, queens are genetically immortal. It is known that sex-asymmetric inbreeding leads to female-biased sex allocation in AQS species (Kobayashi et al. 2013), although the mechanism regulating alate sex ratios remains to be explored. Our model implies that genomic imprinting influences nymph differentiation of male and female offspring, which can act as an important factor regulating sex allocation. Further studies enabling us to assign more precise quantitative values to the parameters would contribute to our understanding of the mechanisms underlying the evolution of life-history traits including sex ratios.

Our study, together with the growing evidence of nongenetic inheritance or parent-of-origin effect in many contexts in various organisms including the eusocial Hymenoptera (Oldroyd et al. 2014; Kocher et al. 2015; Galbraith et al. 2016), calls for a reevaluation of existing data on genetic caste

determination from the viewpoint of genomic imprinting effects in social insects. Our findings lay the groundwork for further investigation of the role of genomic imprinting on the developmental fate of offspring across the social insects.

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King and queen of a termite *Reticulitermes speratus*. Photo credit: Kenji Matsuura.