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Multiple Male and Female Reproductive Strategies and the Presence of a Polyandric Mating System in the Termite *Reticulitermes labralis* (Isoptera: Rhinotermitidae)

J. Wu, X. Su, X. Kong, M. Liu, L. Xing

College of Life Sciences, Northwest University, Xi'an, Shaanxi Province, China.

Corresponding author

Xing Lian-xi
College of Life Sciences
Northwest University
No. 229, North Taibai Rd., Xi'an
Shaanxi Province, 710069, P. R. China
E-Mail: lxxing@nwu.edu.cn

Abstract

Reproductive systems of termite colonies may involve the number of individuals in the reproductive caste and the copulatory selectivity of reproductive individuals (i.e., polyandry or polygamy), both of them impacting directly the fertility and genetic diversity of the colony. Polygamy is widespread in the lower termites, whereas polyandry appears to be mostly absent in termites. In this paper, the differentiation of male and female neotenics was observed in orphaned experimental colonies of the subterranean termite *Reticulitermes labralis.* The artificial orphaned colonies began to produce neotenics only a week after colony establishing, with more neotenics appearing in the same group as time went by. Finally, each experimental group reserved multi-neotenics consisting of male and female neotenic individuals. Our results demonstrated that these neotenic individuals retained in the colony participated in reproduction. A genetic analysis at four microsatellite loci showed that in addition to the conspicuous morphologically male reproductives, there were inconspicuous males or workers that had copulated with the females in the orphaned colony. Multiple male and female reproductive individuals existed together in a single colony, and one female neotenic could mate with several male reproductives in a short time. Thus, multiple male and female reproductive systems and a polyandric mating system are present in *R. labralis*.

Introduction

High fertility of the reproductive caste is essential for maintaining and expanding a termite colony. The most important factors for fertility of termites are thought to be the number of individuals in the reproductive caste and the copulatory selectivity of individuals in the breeding system. Life history and reproductive strategy often differ from one species to another (Lainé & Wright, 2003). The diverse and flexible breeding systems found in lower termites pre-adapt them to invade new environments or marginal habitats (Dronnet et al., 2005). Therefore, the study of the mating systems of termites has important significance to understand colony dispersal and establishment.

In general, termite colonies are founded by a single primary king and a single primary queen that pair during nuptial flights, mate and subsequently produce the other colony members (Thorne et al., 1999). Since the reproductive system of termite is usually monogamous, the fecundity of the colony is relatively weak during the initial establishment of the colony (Thorne, 1998; Ishitani & Maekawa, 2010; Maekawa et al., 2010). In many termite species, abundant neotenics appear in colonies when the primary reproductives die, and take over the reproductive function to maintain the colonies (Hayashi et al., 2003). Some species even differentiate neotenics in the presence of primary reproductives and serve as supplementary reproductives (Roisin, 2000; Grube & Forschler, 2004).

In *Reticulitermes* species, all castes are made up of individuals from both sexes (Snyder, 1926; Lainé & Wright, 2003). Field colonies of *Reticulitermes* have often been found to contain a large number of neotenics, whose sex ratio is strongly female biased (Howard & Haverty, 1980; Vargo et al., 2012). Recently, a new breeding system named asexual queen succession (AQS) was confirmed in *R. speratus* and *R. virginicus*. These species undergo typical colony founding by a pair of primary reproductives. Relatively early in the colony life cycle, the primary queen is replaced by numerous secondary queens (brachypterous female neotenics) that are produced asexually

by the primary queen. These neotenic queens mate with the primary king and produce workers, soldiers and new primary reproductives through sexual reproduction (Matsuura et al., 2009; Vargo et al., 2012). In other words, it is a multiple female and single male reproductive breeding system. Although inconspicuous male reproductive was found in orphaned colonies in laboratory (Fujita & Watanabe, 2010), polyandry appears to be mostly absent in termites (Hartke & Baer, 2011). *R. labralis* is one of the most important economic termite species in central China, especially in urban areas. The main developmental pathway of this subterranean termite forks into two as other *Reticulitermes* species (Takematsu, 1992), following two larval instars (L1 and L2) (hereafter for terminology of termite castes we would stick to Thorne, 1996). One pathway is the line of nymphs equipped with wing buds on the thorax, comprising six instars (N1-N6). The N6 nymphs can molt into alates (imagos). The other pathway is the line of apterous workers, where five successive instars (W1-W5) can be recognized morphologically. Our early study presented three types of conspicuous male supplementary reproductives in *R. labralis*, ergatoids, nymphoids and adultoid form reproductives (floppy winged form and stay in the natal colony as supplementary reproductives after lost their wings) (Xing LX unpubl. data), which were differentiated from W4-W5 workers, N4–N6 nymphs and N6 nymphs, respectively. The purpose of the present study is to investigate the inconspicuous male reproductives and the mating system of small orphaned groups of the *R. labralis*.

Material and Methods

Termites

A mature colony of *R. labralis* was collected in Da xingshan temple, Xi'an, Shaanxi Province, China, in April 2012, and experimental colonies were set up from this colony. Ten experimental colonies were established containing all castes except larvae (1-2 instar juvenile) and neotenics. Each experimental colony was composed of 100 individuals (20 N4-N6 nymphs, two soldiers and 78 W5 workers), being placed into a 120 ml transparent vial with moistened filter paper and pine wood at 20-26ºC in constant darkness. Water was supplied regularly. We were carrying out observations once every two days in the first week, and recorded the number and time of reproductives differentiated in every experimental colony. During the second week until the period of larva appearance in the majority of colonies, we were observing every day and recorded the sex, number and time of differentiated reproductives. The experimental colonies were dissected after 60 days. All female and half of the male neotenics were fixed in Bouin's fluid at 4ºC for 24h, and the other half of male neotenics that had conspicuous morphological characteristics were selected for the subsequent experiment.

Neotenics (nymphoids, ergatoids and adultoid form neotenics) of *R. labralis* significantly differ in external morphology from those of workers or nymphs (i.e. a longer abdomen, darker pigmentation, slight sclerotisation and the presence of eyes). Female and male neotenic reproductives can be distinguished by the seventh sternite: the female has an enlarged seventh sternite covering the eighth and ninth sternites, while the male has a seventh sternite similar to the previous sternites. In order to investigate whether the inconspicuous male neotenics existed or not, each conspicuous male neotenic (nymphoid or ergatoid) was placed into a separate container with 40 W5 workers (both male and female) and 10 N6 female nymphs that come from natal colony and reared for two months. We removed the nymphs from the container immediately after the first female neotenic (usually nymphoid) emerged in the colony. Eight duplicates were established. Two months later, the experimental colonies were dissected, and the abdomens of neotenics were soaked and fixed in Bouin's fluid for gonad histological observation, while the heads and thoraxes of neotenics and their offspring were preserved in 100% ethanol for genetic analysis.

Gonad histological observations

The abdomens of neotenics (ten females and six males) were used for histological observation. Paraffin-embedded sections were made and stained with hematoxylin and eosin. Abdomens preserved in Bouin's fluid were dehydrated in increasing concentrations of ethanol, then transferred to xylene, and finally embedded in paraffin. Serial parasagittal sections (7µm thick) were cut using a microtome (MRS80-074 Ikemoto) and stained with hematoxylin and eosin. Tissues on slides were observed using microscopes.

Genetic analysis

Whole genomic DNA was extracted from parental head and thorax and offspring using TIANamp Genomic DNA Kit (Tian Gen biotcch Co. Ltd), respectively, PCR were performed with four pairs of microsatellite primers, Rs68, Rs78, Rs76 (Dronnet et al. 2004) and Rf6-1 (Vargo 2000). The primers were synthesized by Invitrogen Trading (Shanghai) Co., Ltd. The PCR for the genotyping was performed in the 10µl reaction mixture consisting of 0.25 U of thermostable DNA polymerase, 0.1 mM of each dNTP, each 0.4 mM of primer pairs and genomic DNA solution and 1×buffer for Blend Taq (containing 2µM Mg^{2+}). Amplification conditions were 35 cycles of 30s denaturation at 94ºC, 30s annealing at 54ºC and 30s elongation at 72ºC. The PCR products were run on 7.5% polyacrylamide gels (25mA, 5h) and silver stained, then photographed by gel imaging system (Bio-Rad, Beijing Yuanye Co., Ltd.). Microsatellite genotypes were determined in neotenics and their progeny for the above four microsatellite loci. PCR products amplified from each individual were sequenced by the commercial company (Sangon Biotech Shanghai Co., Ltd) to verify subtle difference in the allele size.

Results

Number of neotenics per colony

Neotenics began to emerge shortly after the establishment of artificial orphaned colonies (6.6±1.3 day), and more neotenics produced by the same colony over time. The largest number of neotenics appeared in approximately 12 days, and the average number of neotenics were 14.9±3.1 in a colony. We could observe biting between neotenics, and the injured neotenics were intensively attacked by numerous workers to death. Subsequently, the number of neotenics decreased with the appearance of physogastric neotenics in the colony. Three weeks later, we could hardly see new neotenic differentiation, but the number of neotenics remained relatively stable in the colony (Fig. 1). Finally, each experimental colony contained multiple neotenics of both sex. After 60 days, 1.4±1.0 male
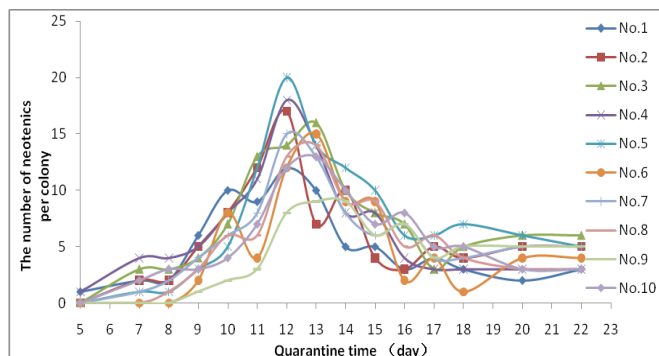

Fig. 1. The fluctuation of *Reticulitermes labralis* neotenics in the 10 orphaned colonies. The first neotenic began to appear after a week, and the number of neotenics became stable after 3 weeks of orphaning. The largest number of neotenic individuals appeared in colonies at approximately 12 days after the establishment of orphaned colonies.

and 3.1±1.1 female neotenics retained in one colony (Table 1). The sex ratio of neotenics was significantly female biased ($p<0.05$), which was consistent with that of field investigations.

Gonad development of neotenics in orphaned colonies

At the end of the experiments, only one colony contained one female neotenic among the 10 colonies; the other nine orphaned colonies had multi-neotenics (Table 1). The ovaries of female neotenics were observed after 60 days. In female neotenics, each ovary was developed and contained plenty of vitellogenic oocytes in the end of the ovarioles, though the number of the vitellogenic oocytes was different among individual neotenics in the colony (Fig. 2A). The observation of spermatogenesis in the testes of male neotenics showed that the testes consumed about half of the abdominal cavity and were full of sperms (Fig. 2B). All the colonies had eggs and offspring at the end of the experiment, which suggested that all the female and male neotenics that eventually survived in the colony participated in reproduction.

Conspicuous and inconspicuous male neotenics in orphaned colonies

In the orphaned colonies, in addition to the male reproductives that had conspicuous morphological characteristics, there were inconspicuous males or workers that participated in mating and reproduction. The eight duplication groups reared in the laboratory were used for genetic analysis. We found that in a 16-offspring colony of the eight duplications, 14 of the 16 offspring were not excluded from further analysis of paternity using the microsatellite assay (Table 2). At the

Table 1 Differentiation of conspicuous neotenics in different orphaned colonies. The neotenics refer to the total number of nymphiods, ergatiods and adultoids in the experiment. * Asterisk denotes significant difference between the average female and male neotenics produced at the end of rearing (Chi-square test, $x^2=5.68 > x^2_{df=1. p=0.05}=3.84$, $p<0.05$).

Colony	Time of first neotenic appearance (day)	Peak number of neotenics in the colony	Number of retained neotenics at the end of rearing	
			Male	Female
1	5	12	2	4
2	6	17	2	3
3	6	16	3	5
4	5	18	0	2
5	7	20	1	3
6	6	15	2	3
7	9	15	1	1
8	8	14	0	4
9	7	9	2	3
10	7	13	1	3
Mean±SD	6.6±1.3	14.9±3.1	1.4±1.0	3.1±1.1*

locus Rs76, the allele sizes of the conspicuous male reproductive were 191/191, but the allele sizes in the offspring (No. 4 and 15) were 175/183 (Fig. 3). Therefore, we can exclude the conspicuous male reproductive from the paternity of these two offspring. In addition to that, offspring No. 4 and No. 15 were a combination of homozygote and heterozygote at the other four microsatellite loci, indicating that they were produced by a mere sexual reproduction. Furthermore, two of the ten orphaned colonies did not emerge to be conspicuous male neotenics (Table 1), but still they produced offspring in the colonies. These data suggested that besides the conspicuous male neotenics, there were inconspicuous males or workers that mated with female neotenics in the orphaned colony.

Discussion

Multiple female neotenics participated in reproduction in a single colony

Neotenics are found in many termite families (Myles, 1999; Roisin, 1999). These neotenic forms appear in termite colonies and take over the reproductive function to maintain the colonies when the primary reproductive individuals die. Sometimes neotenics develop in the presence of primary reproductives as supplementary reproductive individuals (Roisin, 2000; Grube & Forschler, 2004). In mature colonies, female neotenics lay eggs at a lower rate than that of the primary queens. However, they are usually present in large numbers and therefore collectively help produce larger communities even though their individual egg laying capacity is lower (Thorne, 1996, 1998; Thorne et al., 1999). In *R. labralis*, artificially orphaned colonies showed that many neotenics could coexist and laid eggs as secondary reproductives in a colony.

Polygamy is a common phenomenon in termites (Neoh et al., 2010; Hartke & Baer, 2011), and multiple female neotenics are often found in field colonies (Matsuura et al., 2009; Vargo et al., 2012). Although many females could lay eggs in the colony of *R. labralis*, the number of neotenics that retained as secondary reproductive in a colony was limited, which may be influenced by some queen-produced pheromones (Keller & Nonacs, 1993; Yamamoto et al., 2012). The present study showed that *R. labralis* could not only differentiate multiple
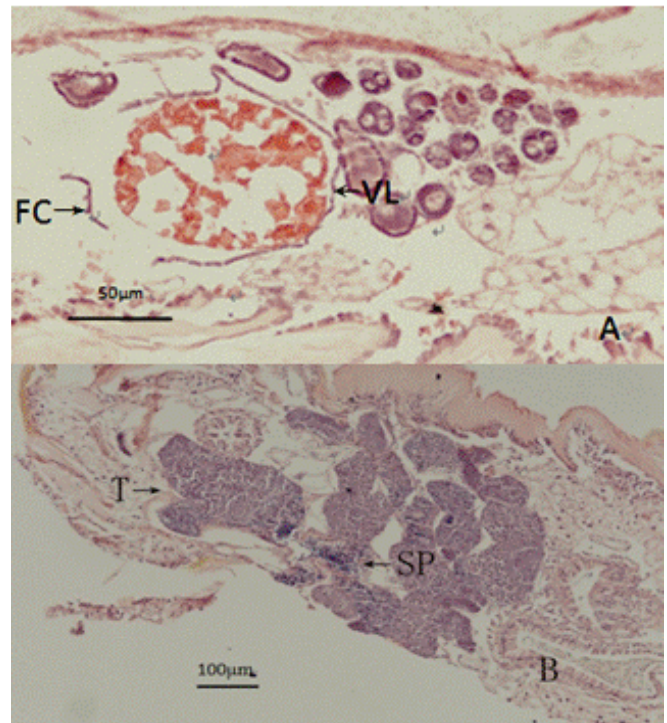

Fig. 2. Parasagittal sections of *Reticulitermes labralis* neotenic gonads at the end of rearing. A: Late vitellogenesis of ovary, the largest vitellogenic oocytes (VL) finished yolk accumulation, which were enveloped by degenerate follicle cells (FC) in the end of the ovarioles. B: The testis of male neotenic, the large number of mature sperm (SP) appeared in testis (T).
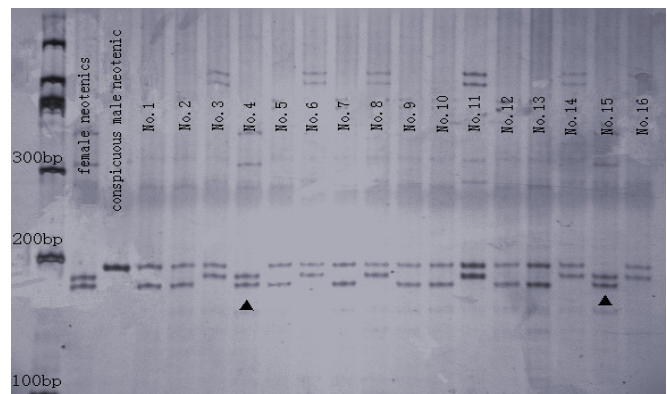

Fig. 3. Genotypes of the conspicuous male neotenics, female neotenics and offspring at the microsatellite locus Rs76. Offspring of No. 4 and No. 15 did not have an allele from the conspicuous male reproductive in their colony. The conspicuous male reproductive was excluded from the paternity of these two offspring.

Table. 2. Allele sizes (bp) and their frequencies (n) of the conspicuous neotenics and offspring. Two offspring (No. 4 and No. 15) were a combination of homozygotic and heterozygotic at the four microsatellite loci, indicating that the individuals were produced by sexual reproduction (*: allele sizes of No. 4. +: allele sizes of No. 15).

	Rs78(n)	Rs76(n)	Rs68(n)	Rf24-2(n)
Male neotenic	178/182(1)	191/191 (1)	165/169 (1)	81/93 (1)
Female neotenic	178/182 (1)	175/183 (1)	165/169 (1)	81/81(1)
Offspring	178/178 (6)+	183/191(6)	165/165 (3)	81/81(9)*
	178/182(7)*	175/191 (8)	165/169 (9)*+	81/93 (7)+
	182/182(3)	175/183 (2)*+	169/169 (5)	

female neotenics laying eggs in a colony, but also produce multiple male neotenics which had distinguishable morphological characteristics. The gonad histological observation suggested that these neotenic individuals all had ability of reproduction. Our field investigation revealed also that multiple female and male neotenics coexisted in the field colonies of *R. labralis* (Xing LX unpubl. data).

Polyandry was confirmed in R. labralis

Reproductive system of termites differ substantially from the one of Hymenoptera insects, where polyandry is common and evolves independently (Boomsma, 1996; Strassmann, 2001; Kronauer et al., 2004; Baer, 2011), and polyandry appears to be mostly absent in termites (Haetke & Baer, 2011). In this study, genetic evidence provided by microsatellite locus Rs76 revealed that two offspring (No. 4 and No. 15) were not paternally related to the conspicuous mature male reproductive of the rearing colony (Fig. 3). Three possible scenarios could explain this result: the female nymph had copulated in her parents' colony before she was orphaned and her spermatheca stored residual spermatozoa, parthenogenic reproduction occurred in *R. labralis*, or there were other male individuals copulating with the female neotenic in addition to the conspicuous mature male.

In contrast to the eusocial hymenopterans, termite copulations occur not during swarming and pair formation but after colony foundation and in the protection of the nest cavity (Nutting, 1969). No sperm has been detected in the spermathecae of female swarming alates (Dean & Gold, 2004; Ye et al., 2009), indicating that these individuals do not copulate within the parental nest during the development of the alates from the nymphs. Our historical observation either found no sperms in the spermathecae of female nymphs and alates in the artificially orphaned colonies (Xing LX unpubl. data). Therefore, it is very likely that the nymphs used in this study did not copulate before the orphaned colonies were established. Although parthenogenesis has been observed in some termite species (Matsuura & Nishida, 2001; Matsuura et al. 2004, 2009; Matsuura & Kobayashi, 2007; Vargo et al., 2012), our literature research found no evidence of the idea that *R. labralis* was capable of parthenogenesis. More importantly, parthenogenesis of termites is accomplished by automixis with terminal fusion, where two haploid pronuclei divide at meiosis fuse (Matsuura et al., 2009). Thus, offspring are homozygous for a single maternal allele at all loci (Bignell et al., 2011; Vargo et al., 2012; Yamamoto & Matsuura, 2012). However, individuals No. 4 and No. 15 in the present study were a combination of homozygote and heterozygote at the four microsatellite loci, thus excluding the possibility of parthenogenesis. The only reasonable explanation for the genotypes of individuals No. 4 and No. 15 is that there were inconspicuous males that copulated with the female neotenic in the experimental colony. In other words, polyandry was present in the subterranean termite of *R. labralis*.

Putting a focus on male neotenics in recent years, some researchers believed that the external morphology of some male neotenics did not differ significantly from those of workers or nymphs (Pichon et al., 2007; Fujita & Watanabe, 2010). These male neotenics were designated as inconspicuous males, or reproductive males. As male reproductive "backups", the presence of inconspicuous males could insure against the loss of male reproductives, and be important for the continued functionality of the termite society. The majority of the reproductive males that are formed do not moult into the neotenic morph and are therefore inconspicuous in the overall population. Indeed, inconspicuous male neotenics and workers have no significant differences in any measured aspect (Fujita & Watanabe, 2010). Workers usually have three developmental pathways: remain workers, become soldiers, or evolve into ergatoid reproductives (Haverty & Howard, 1981). During the observations of *R. labralis*, we found that female neotenics laid eggs in the absence of conspicuous male neotenics in many orphaned colonies. As sperm can be observed both in W5 worker and the last instar of male nymph and male alate (Nutting, 1969; Su et al., 2010; Hartke & Baer, 2011), individuals No. 4 and No. 15 may be the offspring of inconspicuous mature males, but they could also be the offspring of workers, which requires further research. In the subsequent complementary tests, we dissected two replicate colonies at the end of 60-day experiment, one was a conspicuous male ergatoid colony with 28 remaining workers, the other was a conspicuous male ergatoid colony with 22 remaining workers. Histology observation showed that there were 13 male workers in the 28-worker colony, with two workers having significantly bigger testes full of sperms, and nine male workers in the 22-worker colony, with one worker having significantly bigger testes and full of sperms. This confirms unconditionally the existence of sperms in the male worker of *R. labralis*. Regardless of inconspicuous mature males or workers copulate with female neotentics, the present study has confirmed the existence of polyandry in the colony of *R. labralis*.

Reproduction system of R. labralis

The life histories of lower termites are very complicated, and the establishment of a primitive colony requires the nuptial flight, nest building, sexual mating and reproduction (Thorne et al., 1999). Due to the fact that the impact on the reproductive system is usually monogamous, the copulatory selectivity of individuals in the reproductive caste is limited, and fecundity is relatively weak in the initial colony establishment (Snyder, 1926; Thorne, 1998; Ishitani & Maekawa, 2010; Maekawa et al., 2010). When the colonies' populations increase to a certain num-

ber, numerous replacement reproductives appear in termite colonies, thus increasing the number of individuals in the reproductive caste and increasing the total colony reproductive output. Increased reproductive output allows the colony to grow faster and larger because the combined egg production of dozens to hundreds of replacement reproductives far exceeds the egg-laying capacity of a single queen (Thorne et al., 1999; Grube & Forschler, 2004; Vargo et al., 2012). The Japanese species *R. speratus* and the American species *R. virginicus* have been reported to undergo AQS to accelerate the development of the colony, that is, colonies are founded by monogamous pairs and the queens produce their replacements asexually but still continue using normal sexual reproduction processes to produce other colony members (Matsuura et al., 2009; Vargo et al., 2012).

Our results showed that in addition to the presence of numerous female neotenics in the reproductive caste, there were also numerous male reproductives in the *R. labralis* colony at the same time. Moreover, there were inconspicuous males or workers that had the capacity for mating. One female neotenic could mate in a short time with several male reproductives, including inconspicuous males, in spite of the existence of conspicuous males. It is probable that the female neotenics mate with inconspicuous male neotenics to cover potential shortages in the spermatozoa of the conspicuous male neotenics.

The current findings in *R labralis* have greatly expanded the known reproductive repertoire of termites. The individual copulatory options and the flexibility of the mating system are increased by multiple male and female reproductives participating in reproduction in the same colony. The diverse and flexible breeding systems of lower termites pre-adapt them to invade new or marginal habitats (Dronnet et al., 2005). It is likely that multiple male and female reproductives and the polyandric mating system are present not only in *R. labralis*, but in more *Reticulitermes* species as well.

Acknowledgments

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