The origins and relatedness of multiple reproductives in colonies of the termite *Nasutitermes corniger*

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SUMMARY

Colonies of the termite *Nasutitermes corniger* often contain multiple reproductive queens and kings. We used double-strand conformation polymorphism (DSCP) analysis of mitochondrial DNA (mtDNA) to determine the probable origins of co-occurring reproductives. Colonies differed in queen and king number, in the number of nests containing reproductives, and in the genetic relationships among reproductives. Most of the 44 colonies contained a single pair of maternally unrelated reproductives. In the two single-nest colonies with a pair of queens, the two queens differed in mtDNA haplotype, suggesting nest-founding by unrelated queens. In the seven single-nest colonies with larger numbers of reproductives (11–49), all reproductives shared the same haplotype, a pattern consistent with replacement of a single pair by several offspring. As predicted by theory, the number of coexisting queens was greater for replacement reproductives than for co-foundresses. Several complex colonies contained multiple queens of two or more haplotypes distributed among several interconnected nests. This indicates that several matrilineal lines can persist within a colony through one or more generations of budding and replacement, a hypothesis confirmed by orphaning experiments. The various modes of termite colony formation rival the diversity seen in ant species and demonstrate the remarkable convergence of behaviours between the two groups.

1. INTRODUCTION

The number of reproductives within a social insect colony and their genetic relationships are among the most important determinants of colony genetic structure (Wade 1985; Ross 1993). The presence of multiple unrelated queens and kings within colonies is problematic when invoking kin selection as an explanation for the evolution and maintenance of eusociality, because the potential for inclusive fitness benefits appears to be lower in these colonies than in those headed by single pairs (Bourke & Franks 1995; Crozier & Pamilo 1996). Genetic studies on the eusocial Hymenoptera have shown that unrelated queens or multiple insemination of queens can lower relatedness between nestmates and produce conflicts of interest within colonies (reviewed in Keller 1993). Comparable studies on termites, an independent lineage of eusocial insects, have not previously been performed. The typical termite colony is founded by a single queen and king, so that the colony is a simple family. However, multiple queens (polygyny) and kings (polyandry) have been observed in colonies of several species (reviewed in Thorne 1985; Roisin 1993). It is not yet known what effects multiple reproductives have on the genetic structure of colonies. In most documented cases, the origins of the multiple

reproductives are not clear (Roisin 1993), and cannot be determined by morphology. A few genetic studies on termites suggest that colonies are not always simple families (e.g. Reilly 1987; Broughton 1995; Kaib *et al.* 1996), but interpretations are difficult unless colony boundaries are delimited and the colonies' reproductives are collected.

Multiple reproductives can arise in termite colonies by at least two mechanisms, which are subject to different evolutionary pressures and which have different effects on the relatedness of colony members. First, multiple queens and kings may join together following mating flights, a process known as 'pleometrosis'. These original founders are known as 'primary reproductives'. The evolution of pleometrosis requires strong ecological and demographic benefits resulting from increased queen numbers (Thorne 1984, 1985; Roisin 1987, 1993; Nonacs 1989). As in the ants (Keller 1993), confounding termite reproductives may be unrelated, producing varying degrees of relatedness among their offspring. Alternatively, replacement or supplementary reproductives may be reared within the nest following the death of the primary reproductives or during the formation of nest buds (Thorne 1984; Roisin 1993). These 'secondary reproductives' are sons and daughters of the original queens and kings or of later generations of replacements. In contrast to pleometrosis, models of secondary polygyny emphasize kin selection, rather than mutualism, as the driving force (Roisin 1987, 1993). Furthermore,

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production of replacement reproductives by inbreeding increases the degree of relatedness among nestmates (Bartz 1979; Roisin 1993). Other routes to polygyny or polyandry, including the acceptance of unrelated reproductives into existing colonies and colony fusion, are not thought to occur in most termites (Roisin 1993; but see Leponce *et al.* 1996) and are less likely in termites that show aggression toward non-nestmates (Thorne & Haverty 1991).

Studies on the termite genus *Nasutitermes* have invoked both pleometrosis and secondary reproduction as mechanisms producing polygyny and polyandry, but interpretations are sometimes conflicting (reviewed in Roisin 1993). Pleometrosis has been suggested by tandem-running of multiple reproductives after mating flights (Thorne 1984), and by the presence of multiple primary queens in *N. novarumhebridarum*, a species in which primary and replacement queens are morphologically distinct (Roisin 1993). The appearance of new polygynous nests in areas away from other colonies may indicate pleometrosis (Thorne 1984), but could also be the result of secondary reproduction and budding by mature colonies (Roisin 1993). Pleometrotic colony foundation has also been observed in a few other termite genera (reviewed in Crozier & Pamilo 1996; Roisin 1993), but it is not known whether these associations persist as the colonies mature. Rearing of multiple replacement reproductives has been clearly demonstrated in several *Nasutitermes* species by orphaning experiments (Roisin 1987). Additional evidence for secondary polygyny comes from nests with reproductives that are morphologically incapable of flight (Roisin & Pasteels 1985). However, no previous study has examined the genotypes of co-occurring multiple reproductives.

In this study, we used double-strand conformation polymorphism (DSCP) analysis (Saad *et al.* 1994; Atkinson & Adams 1997) of the mitochondrial DNA (mtDNA) control region and 12S ribosomal RNA gene to determine maternal relatedness among reproductives within colonies of the termite *Nasutitermes corniger*. In *N. corniger*, primary and secondary reproductives cannot be distinguished by morphology, except in rare cases (Thorne & Noirot 1982). However, unrelated primary reproductives can be readily distinguished from related replacements by genetic analyses. DSCP markers allowed us to document the relative frequencies of pleometrosis and secondary reproduction in a population of *N. corniger*, and to outline the sociogenetic structure of this termite's colonies.

2. MATERIAL AND METHODS

(a) Study species

Nasutitermes corniger is common throughout most lowland habitats in Central and northern South America (Nickle & Collins 1992). Each colony constructs one or more conspicuous arboreal nests connected by foraging tunnels within a defended territory (Levings & Adams 1984). The colony reproductives live in royal cells near the centres of the nests. In Panama, alate flights take place at the beginning of the rainy season in April and May. The distribution of queen

and king numbers in *N. corniger* has been studied extensively by Thorne (1984, 1985).

(b) Collection

From February to April of 1994 and 1995, we collected *N. corniger* colonies in mangrove forests and secondary growth habitats around Colon, Panama City, Portobelo and the Smithsonian Tropical Research Institute's Galeta Island Laboratory in the Republic of Panama. Nests were assigned to colonies by following foraging tunnels and by testing for aggression between workers and soldiers from neighbouring nests (Levings & Adams 1984). Colonies were selected haphazardly but, as a practical necessity, collection was biased against colonies that had numerous or inaccessible nests. With few exceptions, as described below, every nest of each colony was collected and dissected thoroughly to allow collection of all reproductives. In the laboratory, the queens and kings from each nest were immediately removed from the royal cell, measured, weighed, and examined for indications of age (pigmentation, number of antennal segments) and reproductive type (wing scars, eye type; Thorne 1984). Each queen, along with 20 workers, was placed in a Petri dish lined with moist filter paper to assay egg laying. In colonies with more than 25 queens, randomly selected subsamples were assayed. The rest of the nests were refrigerated for approximately 24 h to inactivate the termites. The nest material was then dissected away, and the termites were sifted from the debris through a series of coarse mesh sieves to help ensure that all reproductives were collected. All queens and kings and samples of each caste were frozen in liquid nitrogen at the time of collection and then stored at -80°C until genetic analysis. To examine production of replacement reproductives, we resampled several colonies from which we had removed the reproductives in the previous year. In these cases, the nest fragments containing the remaining termites were returned to the field after the first dissection, and the workers rebuilt nests near their original positions.

(c) Genetic analysis

DNA was extracted from each termite using the Puregene DNA isolation kit (Gentra Systems, Inc.). All kings and queens from each colony were analysed except for those in five nests that contained more than 40 reproductives; in those, a randomly selected subsample of at least 20 reproductives from each was processed. Some workers, nymphs (developing sexuals), and larvae (developing workers and soldiers) from selected nests were also analysed. Maternal relatedness was assayed by DSCP analysis (Saad *et al.* 1994; Atkinson & Adams 1997) of mtDNA. For DSCP analysis, control region mtDNA was amplified by the polymerase chain reaction (see Taylor *et al.* (1993) for details of the primers used), digested with the restriction enzyme *ApoI*, electrophoresed through thin acrylamide gels, and silver-stained to reveal differences in the electrophoretic mobility of the restriction fragments. Sequence analysis confirmed DSCP haplotype differences. Detailed methods are described in Atkinson & Adams (1997).

3. RESULTS AND DISCUSSION

(a) Numbers of queens and kings

All queens and most kings were found together within the royal cells; some kings, which probably fled during dissection, were later found when nest contents were sifted. The maximum numbers of queens and kings found in one nest were 29 and 57, respectively (tables 1 and 2; 212 queens and 301 kings were found in one artificially orphaned nest). The presence of wing scars indicated that the queens and kings had developed as alate reproductives. Reproductive queens and kings were easily distinguished from virgins at the time of collection, because the latter had not completed their development. All queens that were not damaged during nest dissection laid eggs.

We collected all 90 nests from 43 colonies, and sampled several other nests from one large colony that apparently contained more than 52 nests. Most colonies in this study (37 out of 44) contained reproductives in only one nest. Sixteen of the 44 colonies (36%) had multiple queens and 13 (30%) had more than one king. All polyandrous nests were also polygynous (tables 1 and 2). Excluding the colony with more than 52 nests, 53 nests contained queens. Ignoring colony affiliation, the proportions of nests containing queens (0.59; G -test, $G = 0.004$, d.f. = 1, n.s.), multiple queens (0.36; G -test, $G = 0.052$, d.f. = 1, n.s.), and multiple kings (0.23; G -test, $G = 1.96$, d.f. = 1, n.s.) did not differ significantly from those recorded by Thorne (1984). Note that these counts do not include the artificially orphaned colonies; some of these are discussed individually as examples below.

(b) DSCP haplotype distributions among queens and kings within colonies

DSCP analysis revealed 21 mtDNA haplotypes among the reproductives in the 44 natural and six artificially orphaned colonies assayed in this study. Population mtDNA DSCP haplotype frequencies were estimated by calculating per colony frequencies from reproductives' haplotypes and averaging over colonies. Haplotype frequencies (figure 1) were determined exclusively for the 14 haplotypes found in the 32 colonies collected at the principle study site in Galeta because there was evidence of geographically partitioned genetic substructure (unpublished data). The data are presented under the following four categories, according to the number of nests containing queens and the number of queens per nest.

(i) Colonies with one queen and one king

Twenty-eight of the 44 colonies (64%) had a single queen. Monogynous colonies were also monandrous (see also Thorne 1984), although in some cases the king was not recovered (sometimes because of lack of a search). In 15 out of 19 colonies where the king was collected, the queen and king had different mtDNA haplotypes. In the Galeta population, ten out of 13 single-queen colonies had a queen and king with different haplotypes. Based on the Galeta haplotype frequencies

(figure 1), the proportion of colonies in which the kings and queens shared the same haplotype (0.23) was not significantly greater than that expected for random association ($\sum p_i^2 = 0.15$; where p_i is the frequency of haplotype i ; one-tailed binomial test, $p = 0.31$, n.s.). Furthermore, colonies in which the kings and queens shared the same haplotypes appeared to have been newly established due to the small, lightly pigmented queens, nest masses being under 1 kg, and small workers and soldiers that are indicative of young queens (Oster & Wilson 1978). Thus, there is no evidence that these monogamous pairs resulted from replacement reproduction.

(ii) Colonies with two queens in a single nest

Two colonies contained two queens and one or no king in one nest. In both cases, the colony reproductives had different mtDNA haplotypes. This pattern is consistent with independent colony foundation by unrelated queens. The co-occurring queens were similar in size and coloration, indicating that they were probably the same age. Therefore, colony fusion or acceptance of an unrelated queen into an existing colony are unlikely to have caused this haplotype pattern. Associations of unrelated queens were also seen in multiple-nest colonies, described below (table 2). In total, six out of 44 colonies contained queens with differing haplotypes in at least one nest (14% of all colonies, or 38% of polygynous colonies).

(iii) Colonies with larger numbers of reproductives in one nest

Seven colonies (16% of 44 colonies, or 44% of polygynous colonies) contained large numbers of reproductives all in one nest. In each of these colonies, all queens and kings shared the same haplotype (table 1). The probability of finding this arrangement of haplotypes in a colony formed by independent reproductives (pleometrosis) is extremely low (table 1). This haplotype pattern is especially unlikely to be due to pleometrosis in *N. corniger* because large numbers of closely spaced colonies have simultaneous mating flights. The presence of one haplotype among all the reproductives in a colony would best be explained if these individuals were the offspring of the same mother or group of sibling queens. The reproductives probably resulted from replacement, rather than

Figure 1. MtDNA DSCP haplotype frequencies in the Galeta population.

Table 1. *Reproductive and haplotype distributions found in colonies with many queens and kings in a single nest*

colony	queen #	king #	haplotype	probability ^b
309	11	3	all B	4×10^{-9}
328	19	10	all A	2×10^{-18}
335	11 ^a	6 ^a	all B	6×10^{-11}
399	9	2	all I	4×10^{-7}
521	7	10	all H	6×10^{-11}
542	28	21	all B	1×10^{-30}
554	29	3	all B	3×10^{-20}

^a Some reproductives were lost while removing the nest from the tree.

^b The probability, p , of finding x reproductives with the same haplotype by random association is $p = \sum_{i=1}^n p_i^x$ where p_i is the frequency of haplotype i , n is the number of haplotypes in the Galeta population, and x is the number of reproductives in the colony.

budding, because their nests lacked physical and genetic connections to other nests that contained queens.

We found probable replacements of pleometrotic queens as well but these were always in colonies with reproductives in multiple nests, described below. In addition, multiple replacement reproductives were found in artificially orphaned colonies. For example, we removed a queen with haplotype B and a king with haplotype A from a monogamous colony. The next year, the colony contained 41 queens and 49 kings, all with haplotype B. Replacement of pleometrotic queens was also induced by artificial orphaning (see figure 2).

(iv) *Colonies with reproductives in more than one nest*

The haplotype patterns found in the seven colonies that contained reproductives in separate nests were varied (table 2), and in some cases colony formation processes are more difficult to characterize. All seven colonies had multiple queens in at least one nest. In four of the colonies, multiple haplotypes were seen among queens in single nests. Barring fusion or acceptance of queens from outside the colony, this indicates a history of pleometrosis involving from two to six queens (table 2). It may be that pleometrotic or generally polygynous associations tend to produce multiple-nest (polycalic) systems. Polygynous, polycalic colonies have been noted in other termites (Roisin 1987; Roisin *et al.* 1987) and in ants (Pamilo & Rosengren 1984; Keller 1991; Herbers 1993).

Six of the seven multiple-nest, multiple-queen colonies had some uncertain nest assignments based on a lack of visible interconnections, large distances between nests (greater than 30 m), or weak aggressive responses between termites from some nests. Ambiguity of nest assignment was found only in colonies of this type. Aggression was sometimes non-transitive; in other words, termites from two nests fought with one another while neither fought with a third nest (table 2, colonies 516 and 555; see also Roisin *et al.* 1987). There were different types of

Figure 2. Arrangement of nests and reproductives in a multiple-nest colony (colony no. 325). Solid lines between nests indicate that workers and soldiers were not aggressive; dashed lines indicate that mild agitation was seen. The area of each nest in the figure is proportional to nest volume; the largest nest was 140 litres. This colony was resampled one year after removal of the reproductives shown. Nest IV contained 44 queens (haplotypes: 12 A, 13 B, and 19 D) and one king (haplotype B), nest V contained 212 queens and 301 kings (haplotypes: 38% A, 33% B, and 29% D; $n = 48$), and nest VII contained one queen (haplotype B) and one king (haplotype B). Nests I and II contained no reproductives and nests III and VI were not rebuilt.

Table 2. *Distribution of reproductives and haplotypes among nests^a of multiple-nest colonies*

colony	nest	queen #	queen	king #	king haplotype
307	I	2	C, N	1	C
	III	3	C, C, C	0	—
325	I	3	A, B, D	1	B
	II	1	D	1	B
329 ^c	III	1	B	0	—
	I	7	1 B, 6 E	4	1 B, 3 E
516	II	1	D	0 ^b	—
	III	1	C	0 ^b	—
	IV	1	A	0 ^b	—
	V	2	A, A	0 ^b	—
	II	13	all B	57	all B
525	III	3	B, B, H	2	A, B
	VI	19	all A	2	A, C
540	I	1	C	1	A
	III	2	C, C	2	A, E
555	I	2	R, R	1	A
	III	1	B	1	C
555	I	1	M	1	A
	II	1	F	1	F
	III	21	all F	47	all F
	IV	2	F, F	0	—

^a Only the nests that contained reproductives are listed.

^b Field-dissected colonies; no effort was made to find the king(s).

^c Sub-sample of nests from a colony with more than 52 nests.

aggression (severe fighting versus increased inspection, agitation, and clipping of legs; see also Leponce *et al.* 1996). The variation in aggressive responses might reflect differences in individual recognition cues and could result from the genetic diversity in this colony type (Adams 1991). In all cases, the termites were highly aggressive toward individuals in some of the neighbouring nests, indicating that ambiguous aggressive responses were not caused by a general lack of aggression or loss of nestmate discrimination.

Reproductives in different nests within the same colony often shared haplotypes (e.g. figure 2), suggesting colony expansion by budding and replacement. In other colonies, queens in separate nests had distinct haplotypes (table 2, colony 540, parts of colonies 329 and 555). In these cases, it might be tempting to conclude that the aggression tests that were used to assign nests to colonies were misleading. However, DSCP analysis showed that some workers and immature individuals were not the offspring of queens within their nests, but rather matched the haplotypes of queens in other nests of the colony, confirming that the nests were interconnected. For example, colony 329 contained a large number of nests (more than 52) whose workers and soldiers showed no aggression toward one another. DSCP analysis of individuals from one nest (table 2, nest V) revealed that 23 of the 24 larvae had the same haplotype (A) as the nest's two queens but only 17 of the 40 mature workers and 14 out of 24 nymphs had this haplotype. The other workers, nymphs, and a single larva had four haplotypes (B, C, D and E) that matched queen haplotypes found in other nests of this colony (table 2), and one haplotype that was not previously found (M) but which may have been present in queens from other unsampled nests of the colony. Greater mobility of workers and nymphs may explain the haplotype proportion differences between these castes and the larvae (G -test, $G = 20.43$, d.f. = 1, $p < 0.001$).

This type of aggregation, with its intermixed haplotypes, fits the description of a 'supercolony', where workers travel between nests headed by unrelated reproductives but are highly aggressive toward individuals from other colonies outside their assemblage of nests (Crozier & Pamilo 1996). These workers potentially help raise unrelated young. Such aggregations may have been generated by extensive budding of pleometrotic nests, with a minimum of six matriline in colony 329, adoption of reproductives from outside the colony, or colony fusion. This colony type was commonly seen in our sampling areas but was under-sampled in our study because of the large numbers of nests, some of which were inaccessible. A previous study of *N. corniger* documented a colony with 37 interconnected nests (Levings & Adams 1984).

(c) Polygynous colony formation

Our study indicates that both pleometrosis and replacement reproduction within colonies can lead to polygyny in *N. corniger*. Our data on reproductive numbers and relatedness support previous theoretical

predictions. Roisin's models (1987, 1993) of polygynous termite colony formation indicate that pleometrosis should involve small numbers of queens because of the large mutualistic benefits required to overcome the costs of reproductive competition. Furthermore, relatedness among co-founding queens or kings may have little or no impact on their decision to join together (Nonacs 1989; Roisin 1993). Conversely, large numbers of related secondary reproductives are predicted, because inclusive fitness benefits compensate for reductions in direct reproduction or lower the incentive for fighting. Furthermore, the ecological benefits required to favour acceptance of additional queens decline with increasing queen number. As predicted, unrelated pleometrotic queens were found only in small groups, while replacement reproductives co-occurred in large numbers.

There were no indications of associations between social form and haplotype (as in, for example, the ant *Solenopsis invicta*; Shoemaker & Ross 1996) or social form and habitat (e.g. Herbers 1986), except for the lack of large interconnected nest systems in black mangrove forests where tidal action prevents colony expansion to new trees (Adams & Levings 1987; personal observations). Large polygynous colonies with several nests were found adjacent to monogamous colonies occupying single nests. That monogynous colonies can be changed to polygynous colonies by artificial orphaning illustrates the plasticity of social form in *N. corniger*.

4. CONCLUSION

This study provides genetic evidence of pleometrosis and secondary reproduction in a termite species. *N. corniger* colonies display extensive plasticity of social structure that rivals that found in the ants, their counterparts in the eusocial Hymenoptera (reviewed in Keller 1993). While, true to the termite's reputation for inbreeding (Hamilton 1972), colonies produce replacement reproductives, inbred monogamous colonies were uncommon (or non-existent). Furthermore, this study shows that some colonies contain unrelated queens, which can survive to colony maturity so that each queen contributes to the production of sexual offspring. In fact, several maternal lineages can persist through one or more generations of secondary reproduction. The existence of large, complex colonies consisting of individuals with many different maternal lineages is a previously unknown phenomenon in termites that invites further exploration.

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