

Colony genetic organization and colony fusion in the termite *Reticulitermes flavipes* as revealed by foraging patterns over time and space

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Abstract

Temporal and spatial analyses are seldom utilized in the study of colony genetic structure, but they are potentially powerful methods which can yield novel insights into the mechanisms underlying variation in breeding systems. Here we present the results of a study which incorporated both of these dimensions in an examination of genetic structure of subterranean termites in the genus *Reticulitermes* (primarily *R. flavipes*). Most colonies of this species (70%) were simple families apparently headed by outbred primary reproductives, while most of the remaining (27% of the total) colonies contained low effective numbers of moderately inbred reproductives. Mapping the spatial distribution of colony foraging sites over time revealed that despite the high colony density, the absolute foraging boundaries of most *R. flavipes* colonies were persistent and exclusive of other conspecific colonies, which suggests that this species is more territorial than has been implied by laboratory studies of intraspecific aggression. Nevertheless, we found a single colony (3% of all colonies) which contained the offspring of more than two unrelated reproductives. Although other studies have also described subterranean termite colonies with a similarly complex genetic composition, we demonstrate here that such colonies can form under natural conditions via the fusion of whole colonies. This study underscores how repeated sampling from individual colonies over time and space can yield information about colony spatial and genetic structure that cannot be obtained from conventional analyses or sampling methods.

Keywords: breeding system, colony fusion, colony genetic organization, Isoptera, microsatellites, territoriality

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Introduction

Among all the eusocial insects which live in complex societies, few occupy such an important position and yet remain as poorly studied as the termites (Crozier & Pamilo 1996; Thorne *et al.* 1999). The termites should provide a critical comparative system for the study of social evolution within complex societies because they have evolved these traits independently of the Hymenoptera and without haplodiploid sex determination. Nevertheless, both the ecology and social evolution of termites are poorly understood, primarily because of our poor understanding of the social and spatial organization of termite colonies. This lack

of knowledge partly results from the secretive nature of termites, many of which seldom leave the confines of their food source or the protection of enclosed foraging tunnels. This problem is exacerbated in the subterranean termites of the family Rhinotermitidae, in which the colonies of many species not only possess an ephemeral or ill-defined nest site, but may also exhibit highly mobile patterns of foraging. This combination of traits can lead to uncertainty in the definition of colony boundaries, and this in turn can create ambiguity in the characterization of colony genetic structure.

Reticulitermes species are some of the most commonly studied rhinotermitids in the temperate zones, and *R. flavipes* is a relatively well-known member of the genus from eastern North America. Colony excavations (Banks & Snyder 1920; Howard *et al.* 1982; Myles 1999) and molecular

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work (Bulmer *et al.* 2001; Bulmer & Traniello 2002a) have demonstrated that colonies are sometimes headed by a pair of primary (colony-initiating) reproductives, but are more often populated by secondary (neotenic) reproductives, the latter often occurring in large numbers and exhibiting substantial levels of inbreeding (Reilly 1987; Bulmer *et al.* 2001). Nevertheless, two studies using mitochondrial DNA have revealed that colonies sometimes contain multiple mitochondrial haplotypes (Jenkins *et al.* 1999a; Bulmer *et al.* 2001), indicating that multiple reproductives may not always be close relatives. Genetically diverse colonies of this nature have been described from several other termite species, including *R. grassei* (Clément 1981; Clément *et al.* 2001), *Nasutitermes corniger* (Atkinson & Adams 1997) and *Mastotermes darwiniensis* (Goodisman & Crozier 2002). Explaining the formation of these colonies presents a challenge to students of social evolution, in the same way as do highly polygynous or unicolonial ants that contain unrelated or distantly related queens (Bourke & Franks 1995; Keller 1995; Crozier & Pamilo 1996).

The finding that termite colonies can have a complex family structure has been explained by several mechanisms which can be broadly divided into those driven by alate reproductive strategies and those driven by worker foraging strategies. Alate reproductive strategies can lead to the formation of mixed-family colonies if alates initiate colonies in cooperative groups of three or more unrelated individuals, or if they infiltrate mature, unrelated colonies immediately after swarming. The first mechanism seems likely to occur in the termitid *N. corniger* (Atkinson & Adams 1997), but the only relevant data from the lower orders of termites suggests that these mechanisms are not common. Pickens (1934) reported that incipient colonies in *R. hesperus* are readily destroyed by workers from mature colonies, and that the direct introduction of primary reproductives into established colonies also results in their immediate destruction.

Worker foraging strategies can lead to complex family structure when workers from different colonies forage at the same locations, when workers from different colonies share the same tunnel systems, or when mature colonies fuse. All three of these mechanisms have been applied to explain the sporadic appearance of complex family structure in several *Reticulitermes* species (Clément 1986; Matsuura & Nishida 2001), including *R. flavipes* (Jenkins *et al.* 1999b; Bulmer *et al.* 2001; Bulmer & Traniello 2002a). However, the first of these may be more appropriately considered experimental error rather than a termite strategy *per se*. If the foraging sites for neighbouring colonies occur very close to one another, termites from adjacent but distinct tunnel systems can be mistakenly placed in the same sample vial. It is not known how often this type of error may occur in the field, but at least some researchers take explicit steps to avoid it (e.g. Bulmer *et al.* 2001). The latter two mechanisms (tunnel sharing and colony fusion) must

invoke either reduced aggression or a breakdown in the ability to discriminate nestmates from non-nestmates. Although it is difficult to distinguish between these experimentally, researchers have in fact observed weak aggression against non-nestmates in several *Reticulitermes* species (Clément 1986; Grace 1996; Polizzi & Forschler 1999; Matsuura & Nishida 2001; Bulmer & Traniello 2002b) as well as in the rhinotermitid *Coptotermes formosanus* (Chen & Henderson 1997; Husseneder & Grace 2001). Nevertheless, the likelihood that a breakdown in nestmate recognition would result in mixed-family colonies under natural conditions might be expected to depend on both the spatial proximity of colonies to one another, how they interact when they are in contact, and how their foraging patterns may change over time to affect these interactions. A study of colony spatial organization over time would shed considerable light on the likelihood of these mechanisms occurring. Nevertheless, few studies have presented a genetic analysis of termite colonies collected across several years, and these studies did not include a temporally explicit analysis of colony structure.

The primary goal of the current study was therefore to track the foraging locations of a large number of *Reticulitermes* colonies from natural forests over the course of three field seasons (just over 2 years) using previously developed microsatellites (Vargo 2000). Although the time frame over which we sampled was considerably shorter than the potential lifespan of a termite colony, by sampling large numbers of colonies we hoped to document some of the noteworthy changes in colony spatial and genetic structure which may occur during the life of a colony.

Materials and methods

Field collections

In July 2000 a 22 × 22 m plot was established within each of two forests in Raleigh, NC: one at the Schenck Memorial forest (SF) and another approximately 10 km away at the Lake Wheeler research station (LW). The SF plot was created within a natural stand of predominantly upland hardwoods over 50 years old, while the LW plot was created within a younger (20–30 years old) stand consisting of predominantly loblolly pine (*Pinus taeda*). A grid was created in each plot by inserting 40-cm wooden stakes into the ground to a depth of about 15 cm at 2-m intervals, thus sectioning each plot into 121 squares of 4 m² each. These stakes were not only helpful in spatial orientation within the plots, but were also intended to allow more accurate definition of the boundaries of colonies. However, we note that the stakes could have potentially altered the existing foraging patterns which we intended to observe in the first place if they caused termites to radically alter their foraging patterns to exploit these new resources.

For our initial samples termites were collected from each plot once each month from August 2000 through to May 2001. A partial genetic analysis of these samples indicated that colony identity did not change over this time period, so subsequently samples were only collected twice annually (late spring and late summer) until the conclusion of the study in September 2002 (July 2001, September 2001, May 2002 and September 2002). During each collection both the wooden stakes and any naturally occurring wood debris were checked for the presence of termites. Disturbance to all wood was minimized during these collections to disrupt the normal foraging patterns of the termites as little as possible. During the second year of the study (2001), the initial collecting grid was extended by 2–6 m on those edges at which termites had been collected during the past year, to delineate more completely the foraging range of each colony. The location of each active feeding site was recorded on scale maps and its location was marked in the plot with a flag for future reference. At least 10 worker termites were collected from each active feeding site. They were placed into labelled vials of 95% ethanol and these samples were stored in the laboratory at 4 °C. Soldier morphology was used to tentatively identify termites to species (Scheffrahn & Su 1994), and microsatellite loci which express species-specific alleles were used to confirm these identities (Vargo, unpublished data). Voucher specimens from a subset of colonies of *Reticulitermes flavipes*, and specimens from all colonies of *R. virginicus* and *R. hageni*, have been deposited in the North Carolina State University Insect Collection.

Microsatellite genotyping

DNA was isolated and genotypes were obtained from 10 workers from every feeding site within multi-site colonies, and 20 workers from colonies collected at only one site. DNA was isolated from whole termite bodies ($n = 1820$) using the Puregene® DNA isolation kit (Gentra Systems, Inc.). The manufacturer's instructions were followed except that the RNase step was excluded, and the dried DNA pellet was resuspended in 200 µL of 1× TE. Each worker termite was amplified at six microsatellite loci (*Rf* 1-3, *Rf* 5-10, *Rf* 6-1, *Rf* 11-1, *Rf* 15-2 and *Rf* 24-2) in 10-µL volumes according to the protocols outlined in Vargo (2000), except that we reduced the quantity of template DNA from 4.0 µL to 0.4 µL. The fluorescently labelled products were run on 6.5% polyacrylamide gels, and the bands were detected with a Li-Cor 4000 automated sequencer. Allele sizes were obtained for individual termites either with the aid of the program GENE PROFILER v3.56 (Scanalytics, Inc., www.scanalytics.com), or by visual comparison with lanes containing the amplified products of individuals with known genotypes. Vargo (2004a) reported that these six loci show no consistent patterns of linkage

disequilibrium, suggesting that they represent independently assorting Mendelian markers.

Data analysis

Samples of termites collected at different locations or from different time periods at the same location were grouped into the same colony when their genotype distributions were not significantly different from one another at any of the microsatellite loci. The significance level was obtained by performing genotypic tests of differentiation between all pairs of feeding sites from the same plot using the log-likelihood (G) based exact test (Goudet *et al.* 1996) as implemented by the program GENEPOP ON THE WEB (option 3, suboption 4; Raymond and Rousset, <http://wbioimed.curtin.edu.au/genepop/index.html>). This program yielded independent significance estimates for each of the loci, and overall significance was assessed for each comparison after a Bonferroni correction for multiple comparisons based on the number of loci utilized. GENEPOP ON THE WEB was also used to detect patterns of isolation by distance between colonies via Mantel's test ($F_{ST}/1 - F_{ST}$ vs. distance; option 6, suboption 7) (Mantel 1967). This latter test would allow us to determine whether or not colony reproduction by budding was common in these plots.

Colonies were divided into one of three types based on their family structure. 'Simple families' indicated colonies in which the workers exhibited the expected genotype distributions for the offspring of a single pair of reproductives (significance assessed with a G-test combined over all loci). These could be headed either by the original founding pair of reproductives (primary reproductives), by one primary reproductive and one replacement reproductive (neotenic), or by a single pair of neotenic. 'Extended families' were those colonies in which the worker genotypes collectively met two criteria. First, the worker genotypes were inconsistent with those of a simple family either because the observed genotypes could not be produced by a single pair of reproductives (i.e. workers collectively possessed more than four genotypes) or because the observed genotype distributions diverged significantly from those expected for a single pair of reproductives (assessed with a G-test combined over all loci). The workers from these colonies were the offspring of at least three reproductives. The second criterion for workers comprising extended families was that they could have no more than four alleles at any one locus, indicating that all the supernumerary reproductives were the descendants of a single pair of reproductives. 'Mixed-family colonies' were those whose workers collectively possessed five or more alleles at any one microsatellite locus, and thus must be the descendants of two or more unrelated (or distantly related) same-sex reproductives.

Relatedness was estimated among workers within colonies using Queller & Goodnight's (1989) method, as implemented

by the program RELATEDNESS version 5.0.6 (K. F. Goodnight; <http://www.bioc.rice.edu/Keck2.0/labs/>). Standard errors were obtained by jackknifing over colonies, and significance was assessed via one-tailed *t*-tests (d.f. = #loci - 1). The genotypes of the parents in simple-family colonies were reconstructed by examining the worker genotypes, and relatedness was estimated between the reconstructed parents using the same methods outlined above.

Estimated of hierarchical *F*-statistics were made using the analysis of molecular variance procedure (Weir 1996) with the program GENETIC DATA ANALYSIS (Lewis & Zaykin 2001; version 1.0 (d16c) <http://lewis.eeb.uconn.edu/lewishome/software.html>). Confidence intervals were generated by bootstrapping over loci. Because our plots were not significantly differentiated from one another ($F_{ST} = 0.016$, $P > 0.10$) we excluded the site level in our hierarchical analysis.

We compared all our *F*-statistics and relatedness values to the expected values estimated by Thorne *et al.* (1999) under a variety of different breeding systems. We also follow the notation of Thorne *et al.* (1999) for our *F*-statistics, with the subscripts I, C and T representing the individual, colony and total components of genetic variation. F_{IC} is the colony inbreeding coefficient which, although it is computed in the same way as F_{IS} , has no analogous measure in solitary organisms. Nevertheless, F_{IC} can be extremely valuable in dissecting the breeding structure of colonial organisms because it shows a wide range of values, but at the same time assesses very different patterns of genetic structure from the more traditional measures such as relatedness. It is expected to be negative for simple families, will increase towards zero with increasing numbers of reproductives, and will become greater than zero if there is assortative mating among different sets of kings and queens within a colony. Although F_{IT} ordinarily encompasses both inbreeding and population structure, in the current study it is equivalent to the standard inbreeding coefficient F_{IS} since higher level structure was absent. F_{CT} is the differentiation among colonies, and is thus very similar to nestmate relatedness.

Results

Collections

Over the entire course of the study 51 groups of termites were collected at feeding sites in the Lake Wheeler plot (LW), and 121 groups of termites at feeding sites in the Schenck Forest plot (SF). *Reticulitermes flavipes* was the most commonly collected species at our sites (157 out of 172), with fewer than one in 10 samples represented by either *R. virginicus* or *R. hageni* (14 and one sample(s) collected, respectively). Roughly one in five collections (34 of 172) were made from termites feeding at the wooden stakes, and these comprised less than 10% of the total numbers of wooden stakes in the plots. The rarity with

which the stakes were fed upon suggests that their presence did not radically alter the colony spatial and genetic structure which is reported here.

Genetic markers

The six microsatellite loci exhibited characteristics (number of alleles and expected heterozygosity) similar to those reported in Vargo (2000). In the current study an average of 9.7 alleles per locus (range 3–22) was detected, with an average expected heterozygosity of 0.61 (range 0.07–0.91).

Colony boundaries

The exact tests of genotypic differentiation enabled us to delimit the boundaries of eight *Reticulitermes* colonies in the LW plot (seven *R. flavipes* and one *R. virginicus*) and 25 colonies in the SF plot (23 *R. flavipes*, one *R. virginicus*, and one *R. hageni*). The distribution of feeding sites and the boundaries of most colonies are given in Fig. 1 (LW plot) and Fig. 2 (SF plot). In practice, the delineation of colony boundaries was nearly always unambiguous because of the high allelic diversity of the microsatellite loci. A visual comparison of the alleles and genotypes from different samples of termites would reveal either unique alleles or genotypes at multiple loci or completely overlapping genotypes at all the loci. Thus, when we performed tests of differentiation between sites in the same plot it was either found that differentiation between them was significant at four or more of the loci, or it was non-significant at all of the

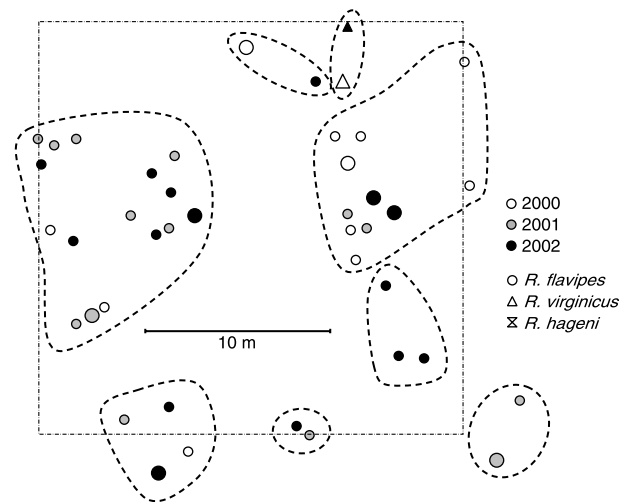


Fig. 1 The distribution of termite colonies in the LW plot over three field seasons. The original plot boundaries are marked with light dashed lines, and the foraging limits of colonies which were collected from multiple foraging sites are marked with heavier dashed lines. The larger symbols mark feeding sites at which termites from a particular colony were collected on at least two separate occasions.

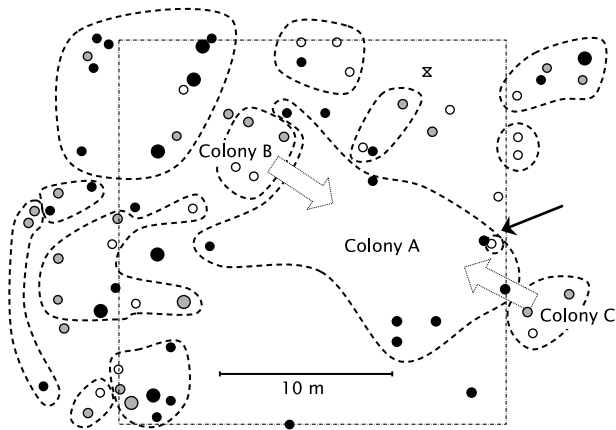


Fig. 2 The distribution of termite colonies in the SF plot over three field seasons, excluding a single colony of *Reticulitermes virginicus* for clarity. Symbol use follows that in Fig. 1. Black arrows highlight those foraging sites at which workers from two separate colonies were collected over the entire course of the study. The large block-style arrows denote the fusion of two previously separate colonies within SF plot (see text).

loci. Significantly differentiated sites were considered to be part of different colonies, while undifferentiated pairs of sites were grouped into the same colony. However, a few exceptional cases did occur, and these could be separated into two categories. The sites in the first such category (four out of 172 sites) were significantly differentiated from some of the sites in a nearby colony, and yet were undifferentiated from other sites within the same colony. These incongruous cases of differentiation were only significant at a single locus, and it appeared to result from differences in genotype frequencies rather than the presence of unique alleles or genotypic combinations. In light of the large numbers of pairwise comparisons made in both plots (over 5000) these ambiguities were attributed to sampling error, and these collection sites were subsequently considered to belong to the colony with which they shared all their genotypes in common. Moreover, actual differentiation within these colonies did not seem likely because two of these four samples appeared to belong to colonies which were simple families during every sampling period, and differentiation over time or space within a single colony would seem likely to occur only in colonies with multiple matrilineal or patrilineal.

In contrast to this first group of sites, a group of eight sites in the centre of the SF plot (colony A in Fig. 2) showed ambiguities in colony boundaries for which we could posit a biologically plausible cause. These sites showed no differentiation between them (thus they were collectively designated as a colony), but showed some genetic similarity to a pair of flanking colonies (colonies B and C in Fig. 2). A visual comparison of the genotypes found in all three colonies showed that the genotypes found in the large,

Table 1 The numbers of each genotype found among workers in colony A and its putative parental colonies (B and C) at the loci *Rf* 5-10 and *Rf* 24-2

Genotypes	Colony		
	B	A	C
<i>Rf</i> 5-10			
147/153	1		
147/159	1	2	
150/150		14	4
150/153		2	9
150/159		17	21
153/153	15	8	
153/159	23	11	
<i>Rf</i> 24-2			
134/182	8	6	
134/188		16	7
134/197		12	9
143/158	1	1	
143/185		3	
158/185	10	7	
158/200	1	1	
179/188		13	9
179/197		9	13
182/185	5	2	
185/200	1	2	

centrally located colony A (collected in 2002) were a nearly perfect union set of the genotypes found in colonies B and C (collected in 2000 and 2001). These genotypes at two loci are shown in Table 1, and this pattern was exhibited by all but one of the six loci. Moreover, in 2002 feeding sites that had only workers from colony B or from colony C were not found, nor was there any statistical indication that worker genotype frequencies changed among the eight foraging sites in colony C. The worker genotypes present in this merged colony also did not show new genotype combinations which could not have been produced in one of the parental colonies (Table 1). It was further noted that workers in the putative parental colonies B and C were not significantly related to one another ($r = -0.11$, $SE = 0.132$).

In general, *R. flavipes* workers from different colonies maintained discrete, nonoverlapping foraging areas. Workers from two different colonies of either species were almost never found simultaneously feeding at the same site, with the obvious exception of colony A from the SF plot which is described above. Moreover, colony boundaries were seldom observed to shift over time in order to envelop those of another colony. Out of the 126 distinct feeding sites, only once was there a turnover of *R. flavipes* colonies at the same feeding site, and even these samples were temporally separated by over 2 years (marked by the black arrow in Fig. 2). The boundaries between *R. flavipes*

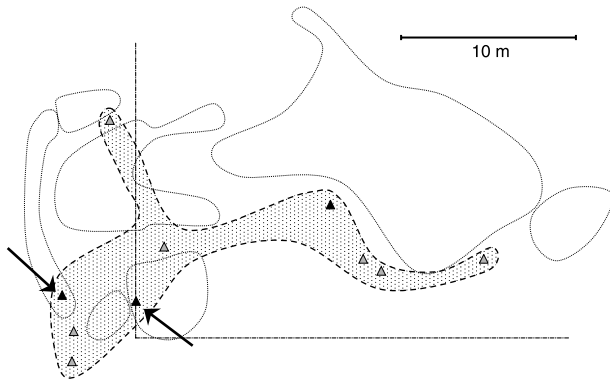


Fig. 3 The shaded area represents the boundary of a single, large *Reticulitermes virginicus* colony in the SF plot. Collecting site symbols follow those in Fig. 1, except that *R. flavipes* colony boundaries are shown without symbols for the sake of clarity.

and *R. virginicus* seemed to be more fluid. One *R. virginicus* colony showed some overlap with the foraging ranges of nearby *R. flavipes* colonies in SF plot (Fig. 3): two of the 10 sites which comprised this *R. virginicus* colony were located in the same pieces of wood which were recently fed upon by extant *R. flavipes* colonies (marked by the black arrows in Fig. 3), a pattern never observed in interactions between different *R. flavipes* colonies.

Colony foraging areas of *R. flavipes* were small. The linear distance separating the two most distant foraging sites utilized by a single colony averaged 5.6 m (range 0.8 m to 17.3 m), excluding colonies which were collected from only a single feeding site. The single *R. hageni* colony was found at only one feeding location, while the distances separating the most widely separated foraging sites in each the two *R. virginicus* colonies were 3.0 m and 23.5 m. However, because the area over which samples were taken

during the course of the study was increased to encompass more fully the colony boundaries, it was not possible to test for changes in colony foraging area over time.

Breeding system. The *R. flavipes* colonies were mostly simple families: 70% (21 of 30) had genotypes consistent with a single pair of reproductives, 27% (eight of 30) were extended families, and 3% (one out of 30) were mixed-family colonies. Although two genetically differentiated groups of workers were in fact mixed-family colonies (colony A and colony B from Fig. 2), we considered them to be a single mixed-family colony A which comprised workers from colony B, but simply sampled at a later date when it also included workers from colony C. Thus, at any one point in time there was only a single mixed-family colony. The *R. virginicus* colony from the LW plot was an extended family, while the large *R. virginicus* colony spanning most of the SF plot was a simple family. The single *R. hageni* colony which we collected in the SF plot was also a simple family. Because of the very small sample of these latter two species, our subsequent analyses were restricted to the *R. flavipes* colonies.

The ability to distinguish mixed-family colonies from extended families by use of the 'four allele rule' will have an error rate equal to the product of the locus-specific probabilities that a single, unrelated reproductive added to a colony will share both of its alleles in common with the residents. The probability of misclassification for the eight *R. flavipes* colonies which were classified as extended families was very low (mean = 0.0057; range 0.0142–0.0001). The probability that we failed to detect mixed-family colonies which arose via colony fusion (by adding two reproductives which both share their alleles with the residents) will be much lower than this value.

F-statistics and relatedness values for the *R. flavipes* colonies are given in Table 2; italics indicate the expected

Table 2 Observed and expected (in italics) *F*-statistics and worker relatedness estimates for *Reticulitermes flavipes* colonies (95% confidence intervals are shown in parentheses), broken down by colony type

	F_{IC}	F_{IT}	F_{CT}	r
Overall	-0.24 (-0.28 to -0.19)	0.12 (0.08–0.16)	0.29 (0.28–0.31)	0.48 (0.46–0.51)
Simple families ($n = 21$)	-0.32 (-0.36 to -0.28)	0.04 (-0.05–0.12)	0.27 (0.21–0.32)	0.52 (0.48–0.55)
<i>Monogamous primary reproductives</i>	-0.33	0.00	0.25	0.50
Extended families ($n = 8$)	-0.14 (-0.22 to -0.08)	0.19 (0.11–0.26)	0.29 (0.22–0.36)	0.46 (0.37–0.55)
<i>One generation of neotenic: $f = 2$, $m = 1$, in a population of 75% simple families</i>	-0.14	0.26	0.35	0.55
Mixed-families ($n = 1$)	0.10 *	* *	* *	0.19 (0.11–0.26)†

*These parameters could not be calculated from a single colony.

†The SE for this confidence interval was obtained by jackknifing over loci.

F -stats for the two colony types which most closely match our own results. The simple-family colonies were not significantly different from the expected results for colonies headed by a single pair of outbred primary reproductives (Thorne *et al.* 1999). The results from the extended-family colonies came reasonably close to the expected values for colonies headed by a trio of first-generation neotenics within a population which is composed of 75% primary-headed colonies and 25% neotenic-headed colonies.

There was no evidence for isolation by distance in either plot. Mantel tests revealed no significant correlation between $F_{ST}/(1 - F_{ST})$ and distance in LW (correlation coefficient = -0.001 , $P = 0.78$) or SF (corr. coeff. = -0.0003 , $P = 0.93$).

Simple and extended families did not differ from one another in several measures of colony foraging area. Simple family colonies did not differ from extended family colonies in either the median numbers of sites at which they foraged (Kruskal–Wallis, $H(\text{adj}) = 0.70$, $P = 0.402$), or in the linear distance over which they foraged (a conservative test which counted single-location colonies as foraging 0.0 m; Kruskal–Wallis, $H(\text{adj}) = 2.27$, $P = 0.132$; excluding single-location colonies $H(\text{adj}) = 0.00$, $P = 0.964$). Nevertheless, considering the extended family colonies alone a highly significant correlation was found between the colony inbreeding coefficient F_{IC} and foraging distance ($r = 0.855$, $P = 0.007$), which was robust to the removal of any colony from the analysis. No such correlation was evident for the simple family colonies ($r = -0.362$, $P = 0.107$) (Fig. 4).

One curious pattern that was noted was the prevalence of ‘singleton’ colonies. These were colonies from which individuals were collected only once during the entire course of the study. These colonies made up a substantial portion of all colonies, 10 of the 30 *R. flavipes* colonies, and all of these occurred in the more densely populated SF plot. There was a marginally significant excess of simple families among these singleton colonies, of which nine out of 10 were simple families, compared to 12 simple families out of the 20 colonies from which individuals were collected more than once (G -test, $G_1 = 3.23$, $P = 0.072$).

Discussion

About two-thirds (70%) of all *Reticulitermes flavipes* colonies were consistent with being outbred simple families, roughly one-quarter (27%) were consistent with being the inbred descendants of a single founding pair (extended families), while the remaining colony (3%) contained the descendants of more than two unrelated reproductives (mixed families). Thus, like in many other termites, the breeding system can differ markedly across colonies in the same population (Clément 1981; Luykx 1993; Atkinson & Adams 1997; Husseneder *et al.* 1999; Jenkins *et al.* 1999b; Bulmer *et al.* 2001; Clément *et al.* 2001; Goodisman & Crozier 2002). Because the simple families were not significantly inbred,

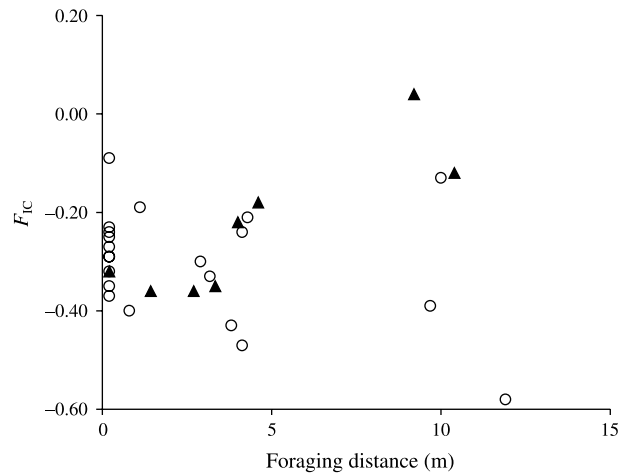


Fig. 4 The relationship between colony inbreeding coefficient (F_{IC}) and the maximum distance separating two foraging sites of the same colony. Data from simple family colonies (○), data from the extended family colonies (▲). The relationship is significantly positive for the extended family colonies (see text).

it can be concluded that most were headed by the original founding pair of primary reproductives. The extended families had inbreeding coefficients consistent with expectation for colonies having low effective numbers of reproductives which have undergone one or a few generations of inbreeding. These results are very similar to those described by Vargo for a study on *R. flavipes* conducted in different locations within these same forests (Vargo 2003a) and in a nearby urban habitat (Vargo 2003b), and this suggests that the addition of extra food sources sites did not significantly alter colony genetic structure. It should be noted that our studies from the populations in North Carolina report a greater proportion of simple families than have been described from other populations of this species; Vargo (2004a) discusses these population-level differences in more detail so they will not be discussed further here.

The presence of a low frequency of mixed-family colonies in North Carolina mirrors what some studies have found in other *R. flavipes* populations (Jenkins *et al.* 1999a; Bulmer *et al.* 2001; Bulmer & Traniello 2002a), and such colonies have also been described in *Mastotermes darwiniensis* (Goodisman & Crozier 2002) and *Nasutitermes corniger* (Atkinson & Adams 1997). Even though these mixed-family colonies seldom appear to constitute a majority of colonies in *R. flavipes* and other termites (but see Clément 1981; Clément *et al.* 2001), their existence is difficult to explain by kin selection theory and therefore deserves exploration. One should point out that relatedness in these colonies remains high enough for sterile workers to achieve some indirect fitness benefits (Goodisman & Crozier 2002). Nevertheless, the conditions under which these colonies form may highlight whether selection favours their formation,

at which level this selection may be acting, and which colony members may or may not be benefiting as a result. These colonies can result from: (i) cooperative colony founding by more than two unrelated alates, (ii) mature colony infiltration by unrelated alates, (iii) mixing of non-nestmate workers at foraging sites, (iv) sharing of foraging tunnels by unrelated neighbouring colonies, or (v) colony fusion. Several of these hypotheses have been applied to explain the formation of mixed-family colonies in the Formicidae (e.g. Foitzik & Heinze 1998; Goodisman & Ross 1998; Heinze *et al.* 2001), but it has been the latter three hypotheses (those in which worker foraging patterns play a primary role) that have been applied most frequently to studies of *Reticulitermes*. The preference for the worker-driven mechanisms arises directly from the observation that *Reticulitermes* workers often fail to express clear patterns of nestmate recognition (Clément 1986; Grace 1996; Polizzi & Forschler 1999; Getty *et al.* 2000; Matsuura & Nishida 2001; Bulmer & Traniello 2002a).

Our results provide the most direct evidence obtained so far that naturally occurring mixed-family colonies can result from the fusion of mature colonies. Two colonies (B and C in Fig. 2) were collected from over 15 m apart in 2000 and 2001, and unique genotypes at five of the six loci indicated that these colonies were not exchanging workers through the autumn of 2001. In the spring and autumn of 2002, workers from both colonies were found together throughout eight foraging sites situated between the two areas previously occupied by the parental colonies. Moreover, all eight foraging sites contained workers from both parental colonies, and genetic differentiation was not detected among these eight sites (seven of which were collected on the same day). The uniform mixture of workers among all eight sites, including one group of 20 workers which was collected 5 months before the other seven sites, indicates that we did not simply mix workers from different tunnel systems into the same sample vials. Moreover, if the putative parental colonies were simply sharing feeding sites or foraging tunnels but were otherwise inhabiting distinct nests, one would not expect the workers to forage in the same proportions over all seven sites collected on the same day, nor would one expect the workers from different colonies to forage in exactly the same proportions among the pairs of sites collected 5 months apart. The most parsimonious explanation is therefore that the two colonies had fused with one another. Nevertheless, interbreeding between reproductives was not detected. One of four hypotheses could explain this lack of interbreeding: (i) mate choice, (ii) spatial separation of the different groups of parents, (iii) the demise of the reproductives from one or both of the colonies, or (iv) that the recombinant offspring were not yet old enough to be sampled. There are currently no data to discriminate between these hypotheses. Also, this fusion event was the only case in which a change in

colony breeding system was found over the course of the study. No other colonies showed changes in genotype frequencies (as indicated by the lack of differentiation within colonies over time) or a systematic increase in the proportion of homozygotes (changes in F_{IC}) over the 25 months of the study. Thus no evidence was found for a turnover of reproductives, suggesting that they typically live longer than the time over which this population was sampled.

Several authors have proposed that abiotic factors have contributed to the alleged breakdown of colony boundaries in other populations of *Reticulitermes*. Clément and colleagues (Clément 1981, 1986; Clément *et al.* 2001) and Bulmer & Traniello (2002a) have hypothesized that soil structure and the ease with which termites can tunnel in that soil have combined with a general lack of agonism to result in the formation of mixed-family colonies. Although colonies must be capable of reaching one another in order to fuse, several observations suggest that soil structure alone probably plays a very minor role in promoting colony fusion. Many colonies from the current study extended to within 1 or 2 m of one another and yet still maintained distinct boundaries without any obvious barriers to tunnelling. Moreover, a concurrent study on *R. flavipes* indicates that colony foraging areas can change radically when some colonies are eliminated through chemical means (Vargo 2003b). This suggests that in these habitats it is not a question of whether foragers come in contact with one another that determines the likelihood that they will form mixed-family colonies, but rather how they interact when their foraging territories share common borders. Some other hypotheses have been forwarded to explain the merger of unrelated colonies in social insects. Some are mechanistic in nature and may invoke changes in odour-cue diversity (Tsutsui *et al.* 2003) or a reduction in social motivation caused by queenlessness (Boulay *et al.* 2003) to explain the fusion of unrelated groups. Matsuura & Nishida (2001) posit that colony merger may have a selective advantage to some colonies, depending upon the composition of the colony with which they merge. However, there are currently no data to test any of these possibilities.

The low frequency of mixed-family colonies at these study sites does, however, indicate that mixing of workers from two different colonies is not a very common occurrence, and that foraging sites are typically only fed upon by workers from a single colony. These fairly static foraging regions suggest that colonies of *R. flavipes* may be more territorial than has been implied by their reluctance to fight with non-nestmates. Territoriality is further supported by the observation that the treatment of colonies with insecticidal baits resulted in a shift of the foraging ranges of the neighbouring colonies to fill the recently abandoned region (Vargo 2003b), as Adams (1990, 2003) has similarly described in territorial ants. The apparently weak agonism in *R. flavipes* (Polizzi & Forschler 1999; Bulmer & Traniello

2002b) could indicate that territorial conflict within this species is resolved through other mechanisms that do not involve overt aggression (e.g. Bagnères *et al.* 1990; Pearce *et al.* 1990; Jmhasly & Leuthold 1999). Alternatively, it may be that aggression itself, which has not been systematically tested in this population, varies geographically and is more pronounced in the current study populations than elsewhere. Territoriality in ants has been linked to the stability and dispersion of resources gathered by colonies (Hölldobler & Lumsden 1980), so one should in fact expect termites, which feed upon the same pieces of wood for several years, to maintain distinct territories that include these resources. Furthermore, interactions between *R. virginicus* and *R. flavipes* did not seem to follow the same pattern: one of the two colonies of the former species appeared to displace foragers of the latter species at two feeding sites, and such feeding site takeover was never observed in any conspecific interactions.

The short distance separating foragers from the same colony (mean 5.6 m) indicates that the foraging areas utilized by single colonies were usually small. No colony from either plot spanned the entire breadth of the plot, and colony density was high in each plot (between five and 14 at any one sampling date). Many studies have reported foraging distances similar in magnitude to those described here (Su *et al.* 1993; Forschler & Ryder 1996; Tsunoda *et al.* 1999; Haverty *et al.* 2000; Bulmer & Traniello 2002a; Vargo 2003a), but in several cases particularly expansive colonies have also been described in *Reticulitermes* spp. (Grace *et al.* 1989; Tsunoda *et al.* 1999; Bulmer & Traniello 2002a) as well as in other termites with a subterranean lifestyle (Su & Scheffrahn 1988; Jones 1990; Husseneder *et al.* 1998; Goodisman & Crozier 2002).

In general our results concur with those of Bulmer & Traniello (2002a) indicating that foraging distances were related to colony genetic structure. However, the particulars of this relationship were somewhat different in the populations studied here. First, no difference was found between simple and extended families in the median distance over which workers foraged, so it can be concluded that colonies headed by outbred primary reproductives forage over spatial distances comparable to those exhibited by neotenic headed colonies. Nevertheless, there was a marginally significant excess of simple families among those colonies which were only active once during the entire survey (singletons). The ephemeral nature of these presumably small simple families probably indicates a high rate of failure for incipient or young colonies.

The other association found here, between colony demography and breeding system was a significant positive correlation between the colony inbreeding coefficient (F_{IC}) and linear foraging distance within the extended family colonies, as Bulmer & Traniello (2002a) also found. The colony inbreeding coefficient is expected to increase

(i) with increasing numbers of neotenic reproductives, (ii) when reproductives occupy spatially separated reproductive centres (or some other process that leads to non-random mating within the colony), or (iii) when there is mixing of workers from different colonies at the same foraging site. The third explanation can be ruled out because it was possible to exclude mixed-family colonies from our analysis. Moreover, since most of the colony inbreeding coefficients were negative, spatially separated reproductive centres could be ruled out as the main cause of this correlation, because this type of spatial structure will yield F_{IC} values greater than zero under a wide range of conditions (Thorne *et al.* 1999). Therefore, it appears that the more expansive extended-family colonies tended to have greater numbers of neotenic reproductives than did smaller extended-family colonies. In contrast, Bulmer & Traniello (2002a) concluded that more expansive colonies in their Massachusetts population must have spatially separated reproductive centres since most of the F_{IC} values for multi-site colonies were above zero. It may be that spatially separated reproductive centres will only arise when the distances separating the feeding sites of a single colony surpass some threshold, an hypothesis consistent with the generally larger foraging ranges Bulmer and Traniello observed in Massachusetts. Also this study did not find any evidence for colony reproduction by budding, as evidenced by the lack of significant isolation by distance among colonies in our forest plots (see also Vargo 2003a). This could result from the high colony densities, the large numbers of relatively young colonies headed by primary reproductives, or the fairly rigid foraging areas associated with a high abundance of food resources.

Conclusion

To the extent that the genus *Reticulitermes* may be representative of termites in general, variation in the colony genetic and spatial organization in termites would seem to be as variable as the better-studied Hymenoptera. The handful of published studies suggest that this variation may even have an important geographical component in at least one species, but more studies which document these patterns over various geographical scales are needed to confirm this observation and determine the mechanisms underlying it. Colonies appeared to forage in a mobile and amoeboid fashion, as others have described (Thorne *et al.* 1999), but when the same colonies were tracked over longer periods of time virtually no overlap was observed of colony foraging areas. Investigating the mechanisms which keep colonies from intersecting or overlapping will not only enhance our knowledge on the foraging ecology of subterranean termites, but may also yield insights into the development of the rare but widespread mixed-family colonies.

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