

Research article

## Strong mitochondrial DNA similarity but low relatedness at microsatellite loci among families within fused colonies of the termite *Reticulitermes flavipes*

C.J. DeHeer<sup>1,2</sup> and E.L. Vargo<sup>1,\*</sup>

<sup>1</sup> Department of Entomology, North Carolina State University, Campus box 7613, Raleigh NC, USA, 27695-7613, e-mail: ed\_vargo@ncsu.edu

<sup>2</sup> Current address: LI-COR, Inc., 4647 Superior Street, P.O. Box 4000, Lincoln NE 68504, USA

**Abstract.** We performed a molecular survey of colonies of the termite *Reticulitermes flavipes* in order to identify colonies which had undergone mergers with one or more colonies of conspecifics, and to provide a detailed description of the breeding system within such fused colonies. By combining the results of previously published studies on *R. flavipes* with unpublished work undertaken for other studies, we compiled a database of 354 colonies from which we identified eight (2.3%) colonies which appeared to have undergone fusion. In the majority of these colonies (six of eight, 75%) we could assign nearly all individuals to one of two (or more) unrelated families ( $r$  between families = 0.016, SE = 0.048,  $P = 0.37$ ), while no individuals had genotypes consistent with interbreeding between cohabiting families. Such intermediate genotypes were also lacking from two colonies sampled during multiple years of the survey, and analysis of immature individuals from these latter colonies further indicated that only one of the cohabiting families had actively reproducing kings or queens. The lack of relatedness between families based on microsatellite markers implies that kin selection does not play a role in the formation of these merged colonies, while the short-lived nature of any increase in genetic diversity suggests that its role is also likely to be minimal. We cannot rule out the hypothesis that some other selective force governs the formation of these mixed-family colonies within the populations that we studied, nor can we exclude the possibility that selection plays a more prominent role in this trait within different populations of *R. flavipes*. Interestingly, we found that merged families had identical or near identical mtDNA haplotypes suggesting a maternally inherited basis to nestmate recognition and colony fusion. Given the lack of detailed information on the recognition system in this termite, we suggest that this phenomenon should serve to encourage further studies on

the mechanisms of recognition within this model termite system.

**Keywords:** Isoptera, Rhinotermitidae, colony breeding structure, kinship, population genetics, nestmate discrimination.

### Introduction

Social insect systems have provided some of the most elegant support for kin selection theory (e.g., Ratnieks, 1988; Sundström, 1994). On the other hand, apparent contradictions to kin selection have also come from these same systems, particularly when their natural history generates unexpectedly diverse patterns of within-colony relatedness (e.g., Crozier and Pamilo, 1996; Beye et al., 1998). Explaining this diversity has been one of the most focused goals in the study of social organisms during the past 20 years. The two predominant mechanisms which generate this diversity are polyandry, multiple mating by reproductive females, and polygyny, the cohabitation of multiple reproductive females within the same colony. Both have been widely studied, and their causes and consequences have been the subject of intense scrutiny (e.g., Keller, 1993; Boomsma and Ratnieks, 1996; Schmid-Hempel and Crozier, 1999). Aside from polyandry and polygyny, several other mechanisms can potentially generate significant within- and between-colony variation in relatedness patterns, including multiple mating by reproductive males and variable patterns of relatedness between same- or opposite-sex nestmate reproductives (Ross, 2001). Although these latter two mechanisms are thought to be rare in the well studied Hymenoptera, they are seemingly common in some groups of social insects (Myles, 1999; Thorne et al., 1999). In spite of being common, however, their effects on

\* Author for correspondence.

patterns of nestmate relatedness appear to be quite subtle (Bulmer et al., 2001; Goodisman and Crozier, 2002; Vargo, 2003a).

In more recent years work in a wide variety of systems has described an additional mechanism which generates more striking variations in patterns of colony genetic structure. This mechanism occurs when two or more colonies merge to form a single entity. Most authors would normally consider this to represent a special case of polygyny, because in effect it results from the adoption of additional queens. However, we consider it separately here in order to highlight the distinct mechanism which generates this family structure, and also because not all cases of colony mergers necessarily result in colonies that contain multiple queens.

Colony fusion has been described sporadically across several groups of social insects. The most striking case of this process arguably occurs in the introduced populations of Argentine ants in which nestmate recognition no longer occurs over ecologically relevant spatial scales, and populations thus appear to have experienced a breakdown of normal colony boundaries (Holway et al., 1998; Giraud et al., 2002; Tsutsui et al., 2003). The resultant colonies may span hundreds or even thousands of kilometers, and this pattern is apparently repeated in other unicolonial, tramp, or invasive species (Holway et al., 2002; Le Breton et al., 2004). Other examples in which colony coalescence results from weakened or absent nestmate discrimination demonstrate that this process can occur on a considerably smaller scale, as demonstrated by mergers of incipient colonies of *Solenopsis invicta* (Tschinkel, 1992; Balas and Adams, 1996) and mature colonies of the acorn dwelling ant *Temnothorax nylanderi* (Foitzik and Heinze, 1998).

Colony fusion has long been a suspected mechanism generating unusual colony genetic structure in termites. Clément (1981), in the first empirical study of termite colony genetic structure, reported a lack of genetic differentiation between colonies of *Reticulitermes grassei* across some regions of France and Spain. Together with this genetic data, a lack of distinct nestmate discrimination in laboratory trials (Clément, 1986) suggested that colony fusion was widespread in some populations. Although recent studies have failed to corroborate these earlier descriptions of widespread colony fusion in this species (DeHeer et al., 2005), Clément's work raised considerable awareness about colony fusion for those working on other subterranean termites. In introduced populations of *Coptotermes formosanus*, inter-colony aggression is often found to be weak or variable (Su and Haverty, 1991; Husseneder and Grace, 2001; Cornelius and Osbrink, 2003), and one mark-release-recapture study described patterns consistent with a colony fusion event (Su and Scheffrahn, 1988). Nevertheless, genetic evidence for colony fusion in this species has remained elusive in spite of the relatively large numbers of colonies which have been assayed (Vargo et al., 2003, 2006b; Husseneder et al., 2005). For *Reticulitermes flavipes*

many experimental results have suggested the possibility that colony boundaries were porous. Laboratory agonism studies (Grace, 1996; Polizzi and Forschler, 1998, 1999; Bulmer and Traniello, 2002; Fisher and Gold, 2003) and field surveys of molecular diversity (Jenkins et al., 1999; Bulmer et al., 2001) both suggested that individuals originating from different colonies may not distinguish between nestmates and non-nestmates, and consequently share the same nests or tunnel systems. However, in contrast to other species of subterranean termites these findings in *R. flavipes* have been confirmed via more direct assessments of colony fusion (Fisher et al., 2004), including one molecular study that provided a time course of colony genotypes before and after merger (DeHeer and Vargo, 2004). Such fused colonies also appear to occur in other groups of termites. Data on colony genetic structure indirectly suggests some frequency of colony fusion among colonies of the monotypic Mastotermitidae (Goodisman and Crozier, 2002), while such colonies occur more definitively in at least two groups of single-piece nesting termites (Thorne et al., 2003; Korb and Schneider, 2007).

The causes of fusion between two or more colonies of social insects may be as diverse as the groups which exhibit this colony structure. As part of the long term goal to fully understand these unusual colonies in *R. flavipes*, we performed a molecular survey of fused colonies in this species in order to both understand the dynamics of colony fusion and to allow us to explore a subset of the hypotheses that can explain this phenomenon. Specifically, these data allowed us to test two genetic hypotheses, which can be generically labeled the relatedness hypothesis and the genetic diversity hypothesis. We note that these are essentially identical to the hypotheses used to explain the evolution of other complex genetic structures like polyandry or polygyny (Crozier and Pamilo, 1996; Schmid-Hempel and Crozier, 1999). The relatedness hypothesis posits that fused colonies are preferentially formed between groups of related termites because any costs associated with fusion are reduced when relatedness remains high in these colonies. This argument necessarily assumes that such colonies experience some general benefits to increasing their size (e.g., Costa and Ross, 2003). The genetic diversity hypothesis posits that increased genetic diversity provides sufficient group-level benefits to offset the costs of decreased relatedness within colonies. The specific benefits of increased genetic diversity could include the same types of benefits hypothesized to occur elsewhere (Schmid-Hempel and Crozier, 1999), in addition to the potential benefit that could result from a reduction in inbreeding. Our current goal is to test whether the genetic signatures present within these fused colonies are consistent with either of these hypotheses. The relatedness hypothesis predicts positive relatedness between colonies that have undergone fusion. The genetic diversity hypothesis does not necessarily predict any relationship between relatedness and the propensity to fuse, other than the obvious

prediction that relatedness will necessarily decrease with an increase in genetic diversity. However, this hypothesis does predict that any increase in genetic diversity should be relatively persistent.

## Materials and methods

### *Detection of fused colonies*

In order to detect colonies that had undergone fusion, we made collections of termites from large numbers of established colonies of *R. flavipes* between May 2000 and October 2004. These collections were originally planned as part of a series of different studies in the Vargo lab, and consequently occurred across multiple locations and in different habitats (see Table 1). At each location, we collected a minimum of 20 adult workers in 95% ethanol from multiple feeding sites, and mapped the locations of these feeding sites relative to one another using compass and measuring tape (e.g., Vargo, 2003a; DeHeer and Vargo, 2004). Samples were stored in alcohol at 4°C until DNA isolations could be performed. During collections, special care was taken to ensure that termites from separate colonies were not mixed into the same vials by 1) always checking the collecting aspirator between colonies, and 2) only collecting termites from a single, short section of log from each colony (distant tunnels in the same log could in theory contain distinct colonies).

We isolated DNA from whole bodies of 20 adult workers from each colony using either the DNeasy Tissue Kit (Qiagen, Valencia CA) or the Puregene® DNA isolation kit (Gentra Systems Inc., Minneapolis MN), following established protocols (e.g., DeHeer and Vargo, 2006; Vargo and Carlson, 2006). In order to identify colonies which may have resulted from the fusion of two or more previously independent colonies, we obtained genotypes from 20 workers at each collection point, using six microsatellite loci (*Rf1-3*, *Rf5-10*, *Rf6-1*, *Rf15-2*, *Rf21-1*, and *Rf24-2*) (Vargo, 2000). These genotype data were first used to delimit the boundaries of colonies, as the same colony may have fed upon more than one collection point within each population. For this we estimated pairwise  $F_{ST}$  between all pairs of sympatric collection points using Fstat 2.9.3.2 (Goudet, 2002), and determined the significance of these estimates by permuting genotypes among samples. If  $F_{ST}$  between groups of workers collected at different locations was significantly greater than zero, then we considered these groups to belong to different colonies. These microsatellite data have been in part presented elsewhere (Vargo, 2003a; DeHeer and Vargo, 2004; Vargo and Carlson, 2006), so we refer the readers to these previous publications for details of PCR, gel running and scoring, and also for details on the breeding system and distribution of the non-fused colonies which formed the majority in these populations.

We also used the microsatellite data to identify colonies that may have resulted from colony fusion. New reproductives are thought to be only recruited from within their own colony in these termites (Myles, 1999), and thus each colony is expected to have at most four alleles per locus (two from each founding parent). Given that most of these microsatellite loci have both large numbers of alleles and high expected heterozygosities (Vargo, 2003a), colonies that have fused together will collectively have more than four alleles at one or more loci, as long as the two colonies did not originate from a single parental colony.

### *Molecular dissection of fused colonies*

We subjected putatively fused colonies to a more in depth analysis in order to obtain a detailed snapshot of family relationships within those colonies. Initially, this involved re-amplifying and re-scoring the first 20 individuals at all loci which exhibited more than four alleles in order to confirm that genotyping errors were not involved. For several of these colonies, we collected 100 or more individuals during our initial collection, and we isolated DNA (as above) from these additional

stored samples in order to bring our sample as close as possible to 100 adult workers per colony.

We obtained genotypes of all available individuals at the six microsatellite loci described above, plus two or more additional loci. The precise identity of these supplementary loci varied depending upon the identity of the population from which the colony originated. Because these different populations were initially investigated by different researchers and during different time periods, the markers used on the rest of the population depended on which of the 16 available microsatellites (Vargo, 2000; Dronnet et al., 2004) was deemed the most useful or the most consistently scored at that time. Although this usually meant that our focal data set of fused colonies shared only six loci in common between every colony, it was statistically more important that both fused and non-fused colonies originating in the same population were scored across the same loci. This is because our primary tool (relatedness estimation) is largely dependent on obtaining accurate allele frequencies from the local population, whereas the allele frequencies within allopatric populations are irrelevant for these calculations (Queller and Goodnight, 1989). Thus, by restricting these additional loci to the same loci already scored in the rest of a population, we avoided the unnecessary task of re-running thousands of individuals at several additional loci. The additional loci that we used included two or more of the following: *Rs 1* (DeHeer et al., 2005), *Rs 10*, *Rs 15*, *Rs 16*, *Rs 62*, *Rs 76*, or *Rs 78* (Dronnet et al., 2004).

In order to obtain additional information on the maternal relationships between cohabiting families within fused colonies, we chose to sequence the cytochrome oxidase II (COII) gene (680 bp) of the mitochondria flanked by the primers A-t Leu and B-t Lys (Liu and Beckenbach, 1992), for which we followed the amplification protocols in Vargo and Carlson (2006). PCR products were cleaned up using the QIAquick PCR purification kit (Qiagen), and amplifications performed using either the ABI Prism dRhodamine Terminator Cycle Sequencing Kit, or the BigDye® Primer Cycle Sequencing Kit (Applied Biosystems, Inc., Forest City, CA). Both forward and reverse reactions were performed on each template following the manufacturers recommended protocol, and dye terminators removed by passage through a CentriSep column (Princeton Separations, Adelphia, NJ). End products were dried in a speed-vac and submitted to the NC State Sequencing facility where they were run on an ABI 377 automated sequencer. We aligned and edited sequences on the Vector NTI Suite package (Invitrogen, Carlsbad, CA), and analyses were performed using Phylip 3.6 (Felsenstein, 2005). A lack of ambiguous positions, particularly from both forward and reverse directions, indicates that these sequences are unlikely to represent nuclear pseudogene copies of the mitochondria (Bensasson et al., 2001). From each putative fused colony we sequenced between two and five individuals per colony. The number and identity of the individuals chosen for sequencing were determined after analyzing microsatellite diversity within each colony, and we obtained sequences from sufficient numbers of individuals to encompass the standing diversity of microsatellite alleles. Thus, if a colony was readily partitioned into distinct families, we obtained mtDNA sequences from one individual per family (Single-family colonies contain only one mitochondrial haplotype; Vargo, 2003a), while we sequenced sufficient individuals to encompass all existing microsatellite diversity within those colonies which could not be partitioned into recognizable family groups. We compared these within-colony differences to the sequence variation found between randomly chosen pairs of colonies that originated from the same population. We limited this analysis to the two populations in which we found three fused colonies (Schenck Forest and the Bentwillow House). Sequences for the Schenck Forest population were taken from previously published work (Vargo, 2003a), and supplemented with 10 additional sequences generated from colonies collected for DeHeer and Vargo (2004). We generated sequences from 10 non-fused colonies collected in the Bentwillow House population.

In addition to obtaining these detailed snapshots of all eight focal colonies, we made collections of termites from two focal colonies over multiple time periods, which allowed us to obtain a dynamic picture of any changes in colony genetic structure that may arise from colony fusion. We were particularly interested in determining whether the reproductives in previously separated colonies would eventually inter-

breed after colony mergers, and in determining the family of origin of new reproductives reared in these colonies. Therefore, from these colonies we not only collected adult workers, but also made a special effort to acquire both immature larvae and nymphs. The latter are immature reproductives which can either become supplementary reproductives within their natal colony, or develop into dispersive reproductives with the potential to found their own colony. We isolated DNA and obtained microsatellite genotypes from a 20–40 individuals during each of these supplementary sampling periods. Basic population genetics analyses were performed using Fstat 2.9.3.2 (Goudet, 2002), and relatedness estimations were performed using Relatedness 5.0.8 (Goodnight, 2004), using the demes option to correct for potential spatial variation in allele frequencies.

## Results

We detected eight putatively fused colonies out of the 354 colonies surveyed (Table 1). Although fused colonies comprised only 2.3 % of colonies across all populations, this frequency varied across populations from 0.0 to 10.7 %. Nevertheless, no two populations from this region differed significantly from one another in the proportion of fused colonies (Fisher's exact test, all  $P > 0.05$ ), so we cannot ascribe any observed spatial variation to true variation in the frequency of fusion across different populations.

**Table 1.** Study locations, sample sizes, and the numbers (and proportion) of fused colonies detected within each surveyed population.

Study location	<i>N</i>	No. fused colonies
Duke Forest <sup>1,4</sup>	36	0
Schenck Forest <sup>1,3,4</sup>	52	3 (0.058)
Lake Wheeler Forest <sup>1,3</sup>	24	0
Blue Ridge Apts. <sup>2</sup>	41	0
NC State campus <sup>5</sup>	10	1 (0.10)
Private homes <sup>6</sup>	146	0
Bentwillow house <sup>6</sup>	28	3 (0.107)
Charles Towne Landing State Historic Site <sup>7</sup>	18	1 (0.055)
Total	354	8 (0.023)

<sup>1</sup>Vargo, 2003a; <sup>2</sup>Vargo, 2003b; <sup>3</sup>DeHeer and Vargo, 2004; <sup>4</sup>Vargo and Carlson, 2006; <sup>5</sup>unpublished data from Vargo lab; <sup>6</sup>Parman and Vargo, in press; <sup>7</sup>Vargo et al., 2006a.

The eight putatively fused colonies could be separated into two classes based on their genetic structure (Table 2). The majority of these colonies (six out of eight, 75 %) matched the isolated examples of fused colonies that we have described previously (DeHeer and Vargo, 2004; Vargo and Carlson, 2006). The average number of alleles at the five most polymorphic loci within these colonies ( $N_a = 5.13$ ,  $SE = 0.37$ ) was considerably higher than the average observed within typical colonies ( $N_a = 2.94$ ,  $SE = 0.09$ ;  $t$ -test,  $t = -9.14$ ,  $df = 30$ ,  $P < 0.0001$ ) (colonies for comparison taken from DeHeer and Vargo, 2004). Also, both the maximum number of alleles observed at

any one locus and the numbers of loci at which we observed more than four alleles were consistent with these six colonies having undergone fusion (Table 2). Finally, one of the more prominent features of genetic structure within these six colonies was the strong association of alleles across loci. For example, in colony sf8 (see Table 3) individuals that have allele 209 at locus *Rf 1–3* are also more likely to have allele 155 or 191 at locus *Rf 6–1*, and allele 217 or 241 at *Rf 21–1*. Although this pattern is statistically similar to what one might find with linkage disequilibrium, these loci do not show this pattern at the population level (Vargo, 2003a). Instead, this type of association at the colony level suggests that colonies consist of two or more readily distinguishable families of termites that have not bred with one another (Table 3). A complete lack of interbreeding is in fact strongly supported in three of these colonies by diagnostic alleles at one or more loci. When the families share no alleles in common at a locus, first generation crosses should have one diagnostic allele from each of the original families, and the lack of such individuals allows us to rule out such crosses as being common. From the remaining three colonies, none of the individuals had genotypes that required inter-family crosses to be explained. Nevertheless, there was a small probability that first generation crosses between reproductives previously inhabiting different colonies escaped detection because of alleles shared between the families. We calculated the expected proportion of these ambiguous individuals as the product of the probability of producing ambiguous genotypes at each locus, which were in turn estimated from family specific allele frequencies. This proportion was low in two colonies (0.0056 and 0.013), but roughly an order of magnitude higher in the third (0.164). Nevertheless, the numbers of individuals genotyped in the latter two colonies (127 and 100 respectively) suggests that we would have detected individuals of mixed ancestry if they were at all common. We lastly note that colony sf8 in Table 3 contained individuals which could not be readily assigned to either of the two other families within the colony, but instead belonged to a third family group. This colony appeared to have undergone fusion multiple times, as did one other colony surveyed (S2). This is consistent with the observation that each of these colonies had at least one locus with more alleles ( $> 8$ ) than could be explained solely by the fusion of two colonies.

The remaining two colonies (25 % of eight) that we tentatively identified as having undergone fusion did not conform to the characteristics described above. The numbers of alleles at the five most polymorphic loci (the same as those used above) were not particularly high in these two colonies ( $N_a = 3.10$ ,  $SE = 0.098$ ), and even though individuals from both colonies were scored at 11 polymorphic loci, each colony expressed more than four alleles at only a single locus which had a modest five alleles. Importantly, we could detect no association between alleles across loci within these two colonies. Thus, sorting workers by their multilocus genotypes did

**Table 2.** Fused colonies identified, numbers of workers analyzed in each colony ( $N$ ), maximum numbers of alleles found at any one locus in each colony (max  $Na$ ), average number of alleles over all loci ( $Na$ ), the numbers of loci with more than 4 alleles present ( $N$  loci 4+), and the number of distinct families found in each colony.

Colony	$N$	max $Na$	$Na$	$N$ loci 4+	distinct families / $N$
nt8a	80	6	4.40	2	yes/2
sf8	80	9	5.40	3	yes/3
S2	100	12	6.80	4	yes/3+
S6	100	7	4.80	3	yes/2
CT3–46	30	7	5.00	3	yes/2
bw11	10	5	4.40	3	yes/2
bw7	35	5	3.20	1	no
bw18	14	5	3.00	1	no

not allow us to separate these colonies into two or more distinct families, and we conclude that the reproductives originating from different colonies were freely interbreeding within these new mergers.

The time course of intensive sampling from two of the typical fused colonies (that is, those which showed no interbreeding in the initial survey) revealed that these colonies also did not experience interbreeding between the families during the subsequent course of sampling. From colony nt8a, from which we sampled twice in 2001 and once in 2002, all of the 91 adult workers and 36 larvae had genotypes consistent with those originally present in one of the two cohabiting families. Although mixed

ancestry individuals could not be entirely ruled out since the families in this colony shared at least one allele in common at all loci, a more definitive pattern was observed in colony sf8. In this later colony, cohabiting families did not share alleles at one locus (Table 3), and mixed ancestry individuals could be ruled out entirely from the adult workers ( $N=390$ ) and larvae ( $N=82$ ) that we sampled over four consecutive years. The larval genotypes from both colonies indicate that the lack of mixed ancestry individuals in both colonies resulted from the death of the parents within one of the founding families. With the exception of a single time period from colony sf8, all the sampled larvae were sired by the two parents of the most abundant family within both colony sf8 and nt8 (Table 4). The exceptional sample was collected in April of 2002, which was only several months after this particular merger occurred (DeHeer and Vargo, 2004). By September of 2002 the new larvae in this colony came exclusively from one family. Not surprisingly, monopolization of reproduction by individuals from only one of the original families resulted in the gradual extinction of the cohabiting, non-reproductive family (Table 4). Unfortunately, very few nymphs were collected for genotyping, and these were all from a single colony (sf8). The genotypes of individuals within this caste were of particular interest as they may develop into either replacement reproductives or winged individuals which leave the nest in order to attempt colony foundation (Myles, 1999; Thorne et al., 1999). Of the two nymphs found in September 2002, one came from each of the two primary families within the colony. The seven nymphs

**Table 3.** Microsatellite genotypes of workers inhabiting a fused colony (sf8) in which no breeding between families occurred. Genotypes outlined dark grey belong to one family, those in light grey belong to a second family (only w4), while those in white belong to a third distinct family.

Individual	<i>Rf 1–3</i>	<i>Rf 6–1</i>	<i>Rf 21–1</i>	<i>Rf 24–2</i>	<i>Rs 10</i>	<i>Rs 78</i>
w1	209/209	155/191	217/241	114/168	152/152	171/171
w2	209/218	152/155	193/241	159/168	149/149	171/179
w3	200/206	152/158	214/226	138/165	149/152	na
w4	197/206	152/158	193/199	165/180	149/149	175/187
w5	209/218	152/155	187/193	159/177	149/152	171/171
w6	209/209	152/152	187/217	159/168	149/152	171/171
w7	209/218	152/152	217/241	114/177	149/149	171/171
w8	200/206	152/158	193/214	114/138	125/152	167/171
w9	206/206	152/152	214/226	114/138	125/149	175/175
w10	200/206	152/152	193/214	162/165	125/149	175/175
w11	209/209	155/191	187/217	159/168	149/149	171/179
w12	209/209	152/191	193/241	159/177	149/152	171/179
w13	200/200	152/152	193/214	138/165	125/149	167/175
w14	200/206	152/158	193/214	114/162	125/149	171/175
w15	209/218	152/191	193/241	159/177	149/149	171/179
w16	200/200	152/158	214/226	162/165	149/149	167/171
w17	209/209	152/155	187/193	159/168	149/149	171/179
w18	209/209	152/152	193/241	114/168	152/152	171/171
w19	209/218	152/191	187/193	159/177	149/149	171/179
w20	209/218	152/155	193/241	114/168	149/152	171/171

collected in September 2004 were all sired by the two extant reproductives, as expected since the developmental split between the worker and nymph line occurs early in larval development (Roisin, 2000).

**Table 4.** The proportion of adult workers and young larvae belonging to the most numerous family within two of the fused colonies which were sampled across multiple years. Sample sizes are given in parentheses.

Colony, caste	2001	2002	2003	2004	2005
nt8, adults	0.88 (62)	1.00 (29)			
nt8, larvae	1.00 (25)	1.00 (11)			
sf8, adults		0.59 (106)	0.69 (114)	0.89 (148)	0.95 (22)
sf8, larvae		0.76 (25)	1.00 (22)	1.00 (17)	1.00 (18)

Microsatellite data were used to generate estimates of relatedness between co-habiting families. Because two colonies had experienced substantial interbreeding between families, we could no longer distinguish individuals from the original families and therefore excluded these from the analysis of relatedness. For baseline allele frequency calculations, we included 20 worker genotypes from each of the non-fused colonies from the sympatric populations surveyed in each location. Because each colony has two contributing diploid genomes (one from each founding parent), these baseline frequencies were based on a minimum haploid number of 68 (17 colonies at Charlestown Landing, each with 4 haploid genomes, see Table 1). However, we deemed the relevant samples obtained from NC State campus as too few for a reliable estimate of local allele frequencies. Thus, for the estimate of relatedness we lumped the fused colony from this population into the same deme as the Schenck Forest population, from which it is less than 5 km distant. Differentiation at this spatial scale is weak or undetectable (Vargo, 2003a; DeHeer and Vargo, 2004; Vargo and Carlson, 2006), but even if present such spatial differentiation would serve to increase our estimate of relatedness slightly, and thus make our estimate conservative. Nevertheless, on average relatedness between distinct families within fused colonies was not significantly greater than zero ( $r=0.0164$ ,  $SE=0.0481$ ,  $SE$  obtained by jackknifing over colonies; one-tailed  $t$ -test,  $t=0.34$ ,  $df=5$ ,  $P=0.37$ ).

In contrast to the microsatellite analyses, mitochondrial sequence data suggest a closer maternal relationship between colonies that have fused with one another. Co-habiting families share very similar haplotypes at the 680 bp region of the COII mitochondrial gene that we sequenced: these families either had identical haplotypes ( $N=5$  family pairs), or differed by only a few base pairs (up to four base pairs;  $N=5$  family pairs). Mitochondrial divergence between randomly chosen colonies from the same population was 0.880% ( $SE=0.150$ ), significantly more than the observed divergence between co-habiting

families (0.164%,  $SE=0.081$ ; Kruskal-Wallis Test,  $X^2=10.2$ ,  $df=1$ ,  $P=0.0014$ ). We thus reject the null hypothesis that colony fusions occur randomly with respect to mitochondrial relationships. We note that these 10 pairwise comparisons were made from only six colonies because two of these contained at least three family groups which allowed multiple pairwise comparisons to be made within some colonies; thus we treat each fusion event as an independent data point. The two putatively fused colonies that lacked clearly distinguishable families also exhibited low mitochondrial diversity. Colony bw7 contained two haplotypes that differed from each other by two base pairs, while all five sequenced individuals in bw18 shared identical COII sequences. Because the latter colony had undergone interbreeding and possible replacement of the original reproductives after the hypothesized fusion event, we cannot rule out a loss of mitochondrial diversity for this and therefore dropped this colony from subsequent analyses. By contrast, even though colony bw7 had undergone reproductive turnover, it still retained different haplotypes and therefore retained at least some of the original diversity at this marker.

## Discussion

The bulk of the evidence presented here suggests that the eight putatively fused colonies did in fact arise via colony fusion. The smoking gun for such a merger has already been presented for one of these colonies (DeHeer and Vargo, 2004), but we note that it is at least possible that multiple alternative scenarios can explain the other seven genetically complex colonies. Given the relative rarity with which we recorded these colonies, human error could cause us to misclassify colonies as fused if even a single individual was scored incorrectly or if collecting errors occurred in the field. We can rule out the former because the initial 20 individuals from each putatively fused colony were always genotyped twice in separate amplification reactions in order to confirm these unusual family structures. An error in field collection could have resulted if individuals from separate colonies were mixed into the same collecting vial, in spite of the special care taken to avoid this. Although errors of this nature are more difficult to rule out, they seem unlikely for two reasons. First, we collected two of these colonies from multiple locations and at multiple time periods, and each respective sample yielded data consistent with all other samples from these locations. Second, the mitochondrial similarity which we describe between these putatively co-habiting families would be difficult to explain by collecting errors, as the colonies most likely to be mixed during sampling (those adjacent to or near one another) are no more likely to have similar mitochondrial haplotypes than are distantly spaced colonies (Vargo, 2003a).

Even if we conclude that human error could not explain these putatively fused colonies, there are other

possible mechanisms that can cause their formation aside from colony fusion, such as mutation or via cooperative colony foundation. We can discount mutation as unlikely because every fused colony was identified as such by at least five individuals, or by at least three different loci. Cooperative colony foundation by more than a single pair of reproductives also cannot readily explain the six colonies that contained clearly distinguishable families which lacked interbreeding between them. We cannot rule out such cooperative founding with absolute certainty for the remaining two colonies, but given the lack of evidence for successful cooperative colony foundation in this genus of termites (Matsuura et al., 2002), the simplest conclusion is that these also represent fused colonies.

The work presented here, in combination with previously published studies (Jenkins et al., 1999; Bulmer et al., 2001), indicates that colony fusion occurs throughout large parts of the range of *R. flavipes*. Nevertheless, the proportion of fused colonies usually remains relatively low where it has been found. Bulmer and colleagues (2001) reported that 13.6% of all colonies in a Massachusetts population potentially represented fused colonies, while we found that the proportion of such colonies ranged between about 5% and 10% at sites where they occurred. Although significant spatial variation in the proportion of fused colonies was not evident from the current study, such a pattern has been suggested to occur among European populations of *R. grassei* and *R. banyulensis*, albeit on a much larger geographic scale and with much greater variation in the proportion of fused colonies (from 0% to at least 60%; Clément, 1981; Clément and Bagnères, 1998; Clément et al., 2001). Clément and colleagues hypothesized that a pattern of spatial variability in the frequency of fusion could result from spatial variability in abiotic factors that can affect the likelihood of colony mergers. Specifically, because soil or climatic characteristics may influence the ability of termite colonies to create and maintain tunnel connections over large distances, the dynamics of colony–colony interactions will vary over the same spatial scales (Clément, 1986; Clément and Bagnères, 1998). Although our results presented here cannot address this hypothesis, the association we found between fusion propensity and mitochondrial similarity suggests that instead of climatic or soil properties, it may be certain colony-level traits that play a role in the formation of *R. flavipes* colonies with extraordinary genetic organization.

One of the obvious findings from the current survey is that the majority of fused colonies do not experience interbreeding between the cohabiting families. Six of eight colonies had workers that could be readily separated into distinct families, with no intermediate genotypes. This suggests either that the reproductives from different families avoid mating with one another, or that one or both families has been orphaned. The genotypic data from two colonies (summarized in Table 4) support the latter hypothesis, but it is not clear whether the orphaning preceded or followed the merger. Comparison

to other systems does not provide a clear prediction for this question. In *Z. angusticollis*, colony fusion results in mutual aggression between reproductives from different families, often resulting in the death of one or more reproductive individuals (Thorne et al., 2003). On the other hand, orphaning could have a causal role in the weakening of colony boundaries; in the single-queen social form of the fire ant *Solenopsis invicta*, loss of the queen leads to a loss of discrimination against non-nestmate individuals (Fletcher and Blum, 1983; Boulay et al., 2003). At first glance, a greater willingness to join with another colony might seem maladaptive for *R. flavipes*, particularly given the apparent ease with which colonies can recruit replacement reproductives from totipotent nymphs or larvae (Thorne et al., 1999). Nevertheless, other selective forces might still favor a strategy incorporating colony fusion under some circumstances (see below).

The analysis of microsatellite data allows us to safely rule out relatedness as an important determinant of colony mergers in these populations of *R. flavipes*, as cohabiting families are not detectably related to one another. This pattern may be unexpected given that genetic similarity has been correlated with inter-colony aggression in some species of ants (Beye et al., 1997, 1998; Tsutsui et al., 2000), and in the termite *C. formosanus* (albeit weakly, Husseneder et al., 2005). It may be that nestmate discrimination simply doesn't have a strong genetic component in this termite. Although cuticular hydrocarbons have been correlated with genetic similarity in *R. santonensis* (= *R. flavipes*, Dronnet et al., 2006) and at least one other termite (Kaib et al., 2004), the critical link between hydrocarbons and nestmate discrimination has not yet been demonstrated for *R. flavipes*. On the other hand, the maintenance of colony boundaries may have little to do with the measures of nestmate discrimination that have been assessed by aggression in this and several other subterranean termite species. Support for this latter hypothesis comes from the observation that studies of colony genetic structure have failed to find evidence for widespread breakdowns in colony boundaries in several species of subterranean termites (Bulmer et al., 2001; DeHeer et al., 2005; Husseneder et al., 2005; Vargo et al., 2006b), even though these species show only weak or at best highly variable levels of aggression against non-nestmate conspecifics (Clément, 1986; Shelton and Grace, 1996; Polizzi and Forschler, 1998; Bulmer and Traniello, 2002; Harahap et al., 2005; Messenger and Su, 2005).

In contrast to the results from nuclear markers, the mitochondrial similarity of co-habiting families was greater than expected by chance. These families had either identical or only slightly different mitochondrial haplotypes (range 0–4 bp differences), whereas randomly chosen pairs of non co-habiting families from the same populations had on average about six times as much mitochondrial divergence (range 0–17 bp). These latter data could suggest that mergers between colonies were

more likely if the families were related through their maternal side. Importantly, the microsatellite data allow us to rule out a close maternal relationship, so we conclude that the mergers occur preferentially between distant maternal relatives. One obvious explanation for this pattern is that these populations are spatially structured over small spatial scales, such that neighboring nests often share similar mtDNA. Although this hypothesis is consistent with the presumed natural history of these termites, which are thought to commonly reproduce via colony budding (Myles, 1999; Thorne et al., 1999), the empirical evidence from a number of studies indicates that these populations lack any molecular signature of colony budding. Isolation by distance, the pattern wherein genetic differences are positively correlated to spatial separation, has not been detected in these populations at any scale under 1 km using either nuclear or mitochondrial genes (Vargo, 2003b; DeHeer and Vargo, 2004; Vargo and Carlson, 2006; Vargo et al., 2006a).

Because we conclude that neighboring colonies are not more likely to share similar mtDNA than non-neighboring colonies, we are left with two general explanations for the similarity of mtDNA haplotypes between co-habiting families within fused colonies. Either something coded on the mitochondria has an influence on nestmate recognition, or such recognition is influenced by factors (possibly symbionts) that are also maternally inherited. In either case, maternal inheritance of nestmate recognition cues would be without precedent, although only one study that we know of has explicitly tested this in termites (in a “higher” termite, Adams, 1991). Interestingly, another study has linked nestmate discrimination to symbiotic gut bacteria in the congener *R. speratus* (Matsuura, 2001). Although the connection here is intriguing, gut symbionts are expected to be inherited biparentally since termite larvae within incipient colonies are fed by both parents. Reasonably convincing proof of this comes from symbiont surveys in mixed species colonies of *Reticulitermes* (Howard et al., 1981). We do note, however, that there are intracellular symbionts of termites that are maternally inherited (Bandi and Sacchi, 2000; Lo and Evans, 2007) and it is possible that these could have an influence on nestmate recognition.

Another observation of potential significance in deciphering the causes of colony fusion is the fact that two of our focal colonies had undergone multiple fusion events. These colonies (sf8, S2) contained worker genotypes from at least three different families, suggesting that these mergers occurred within a few years of one another. Although the reasons for this are obscure, it may be that a greater level of odor cue diversity resulting from the merger may make them more acceptable or more tolerant during subsequent interactions with workers from other colonies, and thus more likely to fuse again (Keller and Passera, 1989; Morel et al., 1990; Matsuura and Nishida, 2001).

Our results here cannot address the full spectrum of the possible causes of colony fusion in this termite, but they do allow us to narrow the range of possible explanations for colony fusion. The relatedness hypothesis does not appear to explain these mergers, as the workers from colonies which merge with one another are no more related to their new nestmates than to the workers from other colonies in the same population. Nor is the genetic diversity hypothesis consistent with the relatively ephemeral increase in genetic diversity which follows most of these colony mergers. Future testing of possible adaptive explanations for colony fusions need not rule out a role for kin selection hypotheses, however. As Matsuura (2001) suggested with his work on *R. speratus*, workers from one colony may allow or disallow mergers depending on the presence of potentially fertile individuals (nymphs) within the other colony, as these will alter the benefits and costs of colony fusion. In the same species, Matsuura (2001) also found evidence for symbiotic bacteria playing some role in the fusion process, and this hypothesis deserves closer scrutiny in other species. Indeed, the significant similarity we find between the mitochondrial haplotypes of co-habiting families within fused colonies of *R. flavipes* could be indicative of a symbiotic organism influencing patterns of colony fusion. We reiterate that in contrast to ordinary gut symbionts, these hypothetical organisms in *R. flavipes* would have to be maternally inherited. Lastly, one must also consider the possibility that the relatively uncommon phenomenon of colony mergers is not a strongly selected trait in these populations, and could simply arise from rare mistakes in nestmate recognition. However, we argue that for two reasons this should not diminish the attention given to the study of this phenomenon. First, it may yield significant insights into a system of nestmate recognition which appears to exist based on studies of colony genetic structure, but which has defied assessment via the direct investigation of mechanisms of recognition. Second, recent data from a variety of systems suggest that genetically complex colonies may be much more common than we describe here. Colony mergers may occur regularly in the life of recently founded colonies of *Zootermopsis nevadensis* (Thorne et al., 2003), and mature fused colonies may comprise 30% of some populations of the drywood termite *Kaloterms secundus* (Korb and Schneider, 2007). We have also found that such colonies may occur more commonly in other populations of *R. flavipes* (DeHeer and Kamble, 2008). We suggest that future work on unraveling the causes of these mergers will benefit from an approach that combines expertise from multiple disciplines, including behavior, chemical ecology, genetics, and perhaps even microbiology.

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