

Christopher J. DeHeer · Edward L. Vargo

An indirect test of inbreeding depression in the termites *Reticulitermes flavipes* and *Reticulitermes virginicus*

Received: 1 April 2005 / Revised: 13 September 2005 / Accepted: 13 October 2005 / Published online: 1 December 2005
© Springer-Verlag 2005

Abstract We analyzed tandem-running pairs of the termites *Reticulitermes flavipes* and *Reticulitermes virginicus* utilizing 13 and 12 microsatellite loci, respectively. Newly formed pairs in both species were significantly related to one another, but this average relatedness was considerably higher in *R. flavipes* (0.130 vs 0.060). These average relatedness levels resulted from some tandem pairs forming between nestmate termites: more than one quarter of all *R. flavipes* pairs (26.1%) met this criterion, while this was the case for only about one of every 20 *R. virginicus* pairs (5.1%). The likelihood that termites paired with siblings was inversely related to the inferred dispersal ability of the two species. F_{ST} , measured over identical spatial scales, was significantly higher in *R. flavipes* (0.034) than in *R. virginicus* (0.008). A comparison in *R. flavipes* of the observed proportion of nestmate pairs observed during tandem running vs the proportion found in established colonies revealed a significant excess of close relatives when pairs were first formed. There are two possible causes of this discrepancy: inbreeding depression (ID) may eliminate inbred colonies early in development, or related pairs may part late in the tandem-running phase or after it is completed. The latter explanation of inbreeding avoidance implies either historical or contemporary ID, and these results therefore suggest that, either directly or indirectly, ID could be a more potent force in the evolution of termite mating systems than is generally appreciated.

Keywords Isoptera · Inbreeding depression · Genetic structure · Microsatellites

Introduction

Inbreeding depression (ID) commonly affects many animal and plant species (Hedrick and Kalinowski 2000; Keller and Waller 2002), with consequences ranging from mating system evolution (Pusey and Wolf 1996; Barrett 1998) to the population viability of endangered species (Hedrick and Kalinowski 2000; Wang et al. 2002). In spite of the costs typically associated with it, inbreeding can nevertheless remain a common component of a species' breeding system (Barrett and Harder 1996; Barrett 1998; Keller and Waller 2002; Henter 2003). In these systems, selection can theoretically purge deleterious recessive alleles, which may reduce or even eliminate negative effects of inbreeding. However, the effectiveness of this purging is seldom complete because the conditions in which it is most effective are not often met in natural populations (Keller and Waller 2002). Therefore, it is perhaps not surprising that species where close inbreeding (or selfing) predominates still exhibit great variability in the strength of ID (Charlesworth and Charlesworth 1987; Byers and Waller 1999). For example, cumulative ID in predominantly selfing plants averages 0.23 (a 23% reduction in fitness), but ranges from slightly negative values (−0.077; outbreeding depression) to substantially positive values (0.568) (Husband and Schemske 1996). On the other hand, outbreeding can also have costs, including the energetic costs of dispersal, an increased risk of predation, and the risk of remaining unmated. The extent to which inbreeding occurs is expected to result from a balance of these opposing forces (Waser et al. 1986).

A cost-and-benefit analysis of inbreeding acquires additional complexity when one considers eusocial organisms. Not only is inbreeding thought to favor the evolution of eusociality in some conditions (Hamilton 1972; Bartz 1979; Michod 1980; Uyenoyama 1984), but group living may in turn entail additional costs and benefits when

Communicated by J. Heinze

This work was funded by grants from the United States Department of Agriculture National Research Initiative Competitive Grants Program (nos. 00-35302-9377 and 2002-35302-12490).

C. J. DeHeer (✉) · E. L. Vargo
Department of Entomology,
North Carolina State University,
Box 7613 Raleigh, NC 27695, USA
e-mail: Chris_Deheer@ncsu.edu
Tel.: +1-919-5153784
Fax: +1-919-5157746

determining the likelihood that inbreeding can be favored. However, in spite of potential consequences that ID may have on evolution within social organisms, the huge literature on ID published during the last two decades (reviewed in Pusey and Wolf 1996; Barrett 1998; Keller and Waller 2002) went virtually unnoticed in the study of eusocial organisms. In part, this may be because ID (via complementary sex determination) was already known in the well-studied Hymenoptera (Cook and Crozier 1995). However, the potential for ID in other social groups, particularly those in which close inbreeding is common, may have great significance for our understanding of two major objectives in the study of social systems: (1) the evolution of sociality, and (2) the evolution of breeding systems and colony genetic structure (Ross 2001).

Inbreeding is thought to be common within several eusocial groups, including naked mole-rats (Jarvis et al. 1994; but see Braude 2000), social spiders (Avilés 1997), and termites (Myles 1999; Thorne et al. 1999). Several noteworthy studies have made at least a rough assessment of the potential for ID in each of these groups. Mate choice studies that demonstrate inbreeding avoidance suggest that mating with relatives may sometimes have costs in at least two of these groups, the naked mole-rats (Ciszek 2000; Braude 2000) and the termite *Zootermopsis nevadensis* (Shellman-Reeve 1996, 2001). However, more direct assessments of ID suggest that net costs to inbreeding may sometimes be negligible. ID was shown to be fairly weak in a subsocial spider (Bilde et al. 2005), while the results from two termite species indicated some costs and some benefits to inbreeding (Fei and Henderson 2003; Husseneder et al. in press), or a significant survivorship benefit to inbreeding with no significant detriment on colony growth (Rosengaus and Traniello 1993). Although these latter results would seem to favor the hypothesis that ID has been mostly purged from some groups of inbreeding social organisms, recent conclusions from works in other systems suggest a more cautious interpretation. In particular, the latter two studies on termites were based on the results of isolated laboratory rearing units, and several reviews (Hedrick and Kalinowski 2000; Keller and Waller 2002) suggest that the fitness advantage of outbred over inbred offspring may only become apparent in the resource-limited, stressful, or otherwise competitive conditions that occur in the wild. Other concerns have been raised regarding experimental design (see Shellman-Reeve 2001), and these suggest that it may be too early to make generalizations about ID in these groups.

Subterranean termites of the genus *Reticulitermes* should provide a promising model system for detailed studies of the potential costs and benefits of inbreeding. The breeding system and colony genetic structure of this genus are particularly well-studied compared to other genera of termites, and inbreeding coefficients show a wide range of variation (Bulmer et al. 2001; Vargo 2003a,b; DeHeer and Vargo 2004; Dronnet et al. 2005; Husseneder et al. in press; DeHeer et al. unpublished data). Inbreeding within termites, in general, can arise through one of two processes:

via pairing of related kings and queens during colony foundation, or via replacement of colony-founding (primary) reproductives with secondary reproductives. Because secondary reproductives are morphologically distinct from primaries, the latter process has been shown to be common in many groups of termites—and *Reticulitermes* are no exception (Myles 1999; Thorne et al. 1999). However, the patterns of relatedness during pair formation have not been well documented. Winged termites are purported to be very weak fliers and have been observed to fly only short distances from their natal nest (Snyder 1935; Nutting 1969; Jones et al. 1988), which might make pairing with nestmates very common. Moreover, because mating flights among different colonies can sometimes be asynchronous, some authors have suggested that colonies are usually initiated by nestmate pairs because unrelated mates are generally unavailable (Snyder 1935; Nutting 1969). In spite of this expectation, the reproductives are nearly always unrelated to one another in *Reticulitermes flavipes* colonies, which contain only one reproductive of each sex (simple families) (Bulmer et al. 2001; Vargo 2003a,b; DeHeer and Vargo 2004). This mismatch between prediction and observation could potentially reflect a selective advantage of outbred pairs during colony initiation. The purpose of the current study is to make a formal assessment of relatedness and genetic structure within newly formed colony-founding pairs of *R. flavipes* and *Reticulitermes virginicus*. By comparing these relatedness estimates to those generated from pairs heading mature colonies, we also make an indirect assessment of ID within these two species of *Reticulitermes*.

Methods

Field collections

We collected pairs of recently flown termites engaged in tandem-running behavior within a 700×100-m section of the North Carolina State University campus during a mating flight in late May 2002 (*R. virginicus*; $n=157$ pairs) and early May 2003 (*R. flavipes*; $n=65$ pairs). Tandem-running pairs are formed by many termite species and usually consist of a heterosexual pair (led by a female), which searches for potential nesting sites immediately after a dispersal flight. At least some level of mate choice has already occurred within these tandem pairs because males must actively follow the female, and the female will usually stop running and assume a calling position if she is not followed by another termite. The collection corridor in which we collected these pairs was a relatively open area consisting of buildings, grassy areas, large trees (mostly *Quercus*), paved pathways, and bricked plazas. For both species, each pair was placed in a separate collection tube, given a label specific to the location in which it was collected, and stored in 95% ethanol at 4°C until subsequent genetic analysis. During these collections, we also made observations on the characteristics of flights in the

two species and repeated these observations in spring 2004 within natural areas to confirm several observations made in the current study.

Microsatellite analyses

We determined the sex of each termite under a dissecting microscope (Pearce 1997) and then immediately isolated DNA from its whole body using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). The resulting dried DNA pellet was resuspended in 300 μ l of 1 \times TE buffer. We genotyped all individuals from both species at 12 microsatellite loci (*Rf1-3*, *Rf6-1*, *Rf21-1*, and *Rf24-2*, Vargo 2000; *Rs1*, DeHeer et al. 2005; *Rs10*, *Rs15*, *Rs16*, *Rs43*, *Rs68*, *Rs76*, and *Rs78*, Dronnet et al. 2004), and *R. flavipes* was also scored at a 13th locus (*Rs62*, Dronnet et al. 2004), which was invariant in *R. virginicus*. All these loci conform to expected patterns of inheritance based on family studies and patterns of genotypic disequilibrium (Vargo 2003a; Vargo, unpublished data). Our amplification protocols followed Vargo (2000), with the following changes. Instead of a generic M13F tail, every primer was individually labeled with either 5' IRD700 or 5' IRD800 fluorescent modifications (MWG-Biotech). We also reduced the number of stutter bands for *Rs78* in *R. virginicus* by amplifying with a more stringent program (a 60°C annealing temperature instead of 54°C). Conversely, we obtained a stronger amplification for *Rf1-3* in both species by using a less stringent program (annealing temperature, 50°C). PCR products were separated by electrophoresis on 6% polyacrylamide gels run on a Li-Cor 4300 DNA analyzer, and we determined allele sizes by comparison to 50- to 350-bp IRDye700 or 800 standard (Li-Cor, Inc.).

Statistical analyses

We used Fstat 2.9.3.2 (<http://www2.unil.ch/izea/software/fstat.html>) to calculate *F* statistics (Weir and Cockerham 1984) separately for each species, as well as separately for males and females within each species. These results allowed us to assess relative dispersal distances within each species and to determine whether dispersal differed between males and females (perhaps as an inbreeding avoidance mechanism; Pusey and Wolf 1996). For *R. flavipes*, the area over which we collected could be naturally divided into three "populations" by virtue of their spatial distribution: termites from the same population were collected closer to one another (<100 m) than were those collected from different populations (100–700 m). Confidence intervals (95%) for point estimates of all *F* statistics were generated by bootstrapping over loci; standard errors were generated by jackknifing over loci. Point estimates were considered significantly greater than zero if their confidence intervals did not overlap zero; significant

differences between point estimates were assessed via approximate *t* tests, which assume unequal variance (Sokal and Rohlf 1981).

We estimated relatedness between termites within tandem pairs using Relatedness 5.0.8 (K.F. Goodnight; <http://www.gsoftnet.us/GSoft.html>), with each population treated as a separate deme for reference allele frequencies (Queller and Goodnight 1989). We obtained standard errors by jackknifing over loci, which allowed the generation of 95% confidence intervals around the population mean estimates of relatedness. Differences from zero were assessed via one-tailed *t* tests (df =number of loci–1), while differences between point estimates were assessed via approximate *t* tests (Sokal and Rohlf 1981).

As a further means to identify pairs of termites that conformed to the expected genotypes of individuals originating from the same colony, we used the likelihood calculations in Kinship 1.3.1 (K.F. Goodnight; <http://www.gsoftnet.us/GSoft.html>). For all comparisons, our null hypothesis was that individuals were unrelated to one another ($r=0$), while our hypothesis was that pairs were nestmates ($r=0.50$ for *R. flavipes* obtained via a weighted average of nestmate relatedness values, Vargo 2003a,b; DeHeer and Vargo 2004; $r=0.50$ for *R. virginicus*, Vargo unpublished data). We note that, contrary to expectations, colony fusion (e.g., DeHeer and Vargo 2004) and inbreeding have little effect on nestmate relatedness because the former is too rare and the latter only affects relatedness in a relatively restricted set of conditions (very few reproductives and several successive generations of inbreeding; Thorne et al. 1999), which do not occur in the species we studied here (Vargo 2003a,b; DeHeer and Vargo 2004; Vargo unpublished data). We performed this Kinship analysis both within tandem-running pairs and between all individuals within each of the three populations independently. This latter test yielded an indirect measure of the number of different colonies contributing to the alate pool of each species. Because Kinship tests involved hundreds or thousands of pairwise comparisons for determining relationships within tandem pairs and within populations, respectively, we used more stringent threshold levels of the significance for these two tests in order to minimize type I errors ($p<0.01$ for tandem-running pairs and pairs within established colonies; $p<0.001$ for population-wide estimates). Traditional corrections for multiple comparisons were not possible because exact *p* values are not available in the Kinship output. However, changing these rule-of-thumb threshold values did not affect the significance of any of the results.

Comparative analysis

In order to determine whether or not highly related tandem pairs perform as well as unrelated pairs during colony founding, we compared the relatedness patterns of these newly formed pairs to the relatedness patterns of those

heading established colonies. If ID is small or negligible, then these values should not differ from one another, but if ID is strong, then one might see a substantial drop in relatedness between young putative colony-founding pairs and those within mature colonies. In order to make this comparison, we estimated relatedness and identified putative nestmate pairs among those heading established colonies of *R. flavipes* and *R. virginicus*. These data comprised the reconstructed parental genotypes of established colonies headed by single pairs of reproductives (simple families), thus most likely representing the genotypes of the primary reproductives that initiated these colonies. However, they may also represent the genotypes of colonies wherein the primary reproductives have been replaced by a single pair of secondary reproductives, which are, by definition, close relatives. As such, this comparative data set could overestimate the numbers of closely related tandem pairs that succeed in founding their own colony; therefore, these comparisons are conservative for the purposes of this study.

Ideally, this comparative analysis should be performed on established colonies from the exact same location where the tandem pairs were collected, in order to control for the possibility that the genetic organization of established colonies varies over these spatial scales. This is unfortunately not practical because the only location in which these recently flown termites are relatively easy to find (sidewalks and plazas; they disappear into leaf litter too quickly in natural areas) is also the location where dead or decaying wood is absent from the surface of the ground. Because such wood (their only food source) is the only location from which sufficient numbers of workers can be found to adequately characterize colony family structure, we have been unable to generate a large genetic data set from established colonies from this area. Instead, for comparative purposes, we used a data set that we compiled from several different studies on established simple-family colonies (only those containing a single pair of reproductives) collected within 15-km of the current study site (Vargo 2003a,b; DeHeer and Vargo 2004; Vargo and Parman unpublished data). For *R. flavipes*, these colonies were collected across three woodland populations ($n=75$ colonies) (Vargo 2003b; DeHeer and Vargo 2004) and 18 private properties ($n=168$ colonies), of which the latter were in close association with human structures (Vargo 2003a; Vargo and Parman, unpublished data). The corresponding analysis on *R. virginicus* was performed on an unpublished data set of simple-family colonies ($n=16$) from the same region (Vargo, unpublished data). We suggest that this comparison across locations is appropriate (at least in *R. flavipes*) by demonstrating two important patterns. First, we show that established colonies of *R. flavipes* show little spatial variation in their genetic organization within this region of North Carolina, regardless of habitat or colony density. Second, genetic data from recently flown termites from the current study site indicate that established colonies therein are quite similar to those from our comparative data set on nearby populations.

Results

Flight observations

We made several observations on the dispersal flights of these species, which, in hindsight, may help explain some of the differences in how they are genetically structured, as described below (Table 1). First, the density of recently flown sexuals of *R. virginicus* was conspicuously higher than that of *R. flavipes*. For the same sampling effort, we collected more than twice as many pairs of the former species ($n=156$ pairs, excluding a single homosexual pair consisting of two males) as the latter ($n=65$ pairs). One possible explanation for this is that *R. flavipes* colonies are simply less common at this site than are *R. virginicus*. However, surveys from nearby locations (Vargo 2003a,b; DeHeer and Vargo 2004; Vargo unpublished data) and a small data set from the current study site ($n=10$ colonies) suggest the opposite: that *R. flavipes* colonies outnumber those of *R. virginicus* by roughly an order of magnitude. Differences in individual density could instead result from a larger colony size in *R. virginicus*, which is suggested by several anecdotal observations in North Carolina and elsewhere (Howard et al. 1982; Vargo 2003a,b; DeHeer and Vargo 2004). In addition, observations suggest that *R. flavipes* may have relatively asynchronous patterns of flight activity that would further decrease the density of individuals during dispersal flights. Our observations of mating flights within natural areas during spring 2004 were performed at a site where the locations of all colonies within a 25×25-m grid have been mapped out previously (DeHeer and Vargo 2004). Within this area known to contain more than a dozen *R. flavipes* colonies at any one time, we observed winged termites emerge from no more than one of these colonies during three separate days of flight activity. Another notable difference between the species was in their emergence and landing locations. *R. virginicus* took flight primarily from near the tops of living trees, usually 15–20 m off the ground, while those of *R. flavipes* emerged directly from the ground or from rotting wood very close to the ground. Moreover, winged *R. virginicus* landed almost exclusively upon the trunks of

Table 1 Comparison of mating flight characteristics in the two studied species

Characteristics	<i>R. virginicus</i>	<i>R. flavipes</i>
Colony density ^a	Low	High
Colony size ^a	Large	Small
Synchronization of flights ^b	High	Low
Individual density ^b	High	Low
Emergence and landing ^b	Arboreal	Terrestrial
Dispersal distance ^c	High	Low
Probability of pairing with nestmate ^c	Low	High

^aBased on Vargo (2003a,b), DeHeer and Vargo (2004), and unpublished data

^bBased on observations from the current study

^cBased on formal statistical analyses from the current study

large living trees, and it was from these vertical surfaces that we collected all tandem-running pairs of this species. In contrast, winged *R. flavipes* always landed and formed tandem pairs on level ground. Moreover, because winged *R. flavipes* emerged from the ground and tended not to fly higher than a few meters, we were able to observe that most individuals tended to alight and remove their wings only a few meters from their nest. We confirmed these observations both in campus collections utilized in the current study and in natural areas.

F statistics

We present the F statistics for both *R. virginicus* and *R. flavipes* in Table 2. F_{IS} , the deviation from random mating within populations, was moderate but significantly greater than zero among the recently flown reproductives of both species. These low but significant values indicate that the majority of new reproductives from both species are produced within outbred colonies, while some substantial minority are produced within colonies headed by close relatives. This is entirely consistent with the observation that 25% of established *R. flavipes* colonies are headed by multiple, highly related replacement reproductives (average of Vargo 2003a,b; DeHeer and Vargo 2004). One unexpected pattern, however, was that recently flown *R. virginicus* showed a significant difference between the sexes in estimates of F_{IS} , with females exhibiting greater F_{IS} than males (two-tailed; $t_s=3.03$, $df=21$, $p=0.006$).

F_{ST} was also significantly greater than zero in both species, although point estimates were relatively low in each case. Nevertheless, *R. flavipes* exhibited significantly larger F_{ST} than did *R. virginicus* (two-tailed; $t_s=3.57$, $df=14$, $p=0.003$). Neither species exhibited sexual dimorphism in estimates of population differentiation (F_{ST}) (two-tailed; *R. flavipes*, $t_s=1.16$, $df=17$, $p=0.26$; *R. virginicus*, $t_s=1.39$, $df=19$, $p=0.18$). We note that the interpretation of significant F_{ST} is somewhat complicated by the use of individuals that have just participated in a dispersal flight. Genetic structure among these populations of tandem pairs will not only result from genetic differentiation among

populations of their natal colonies, but will also reflect both the dispersal distances of alates and the distribution and abundance of colonies that emitted reproductives. Other studied populations of *R. flavipes* do not show genetic structure at distances of <1 km (Vargo 2003b; DeHeer and Vargo 2004); thus, the significant F_{ST} in this species likely results from the latter two processes.

Relatedness and Kinship

In both *R. flavipes* and *R. virginicus*, recently flown sexuals were significantly related to their tandem-running partner (Table 2), but relatedness was higher in *R. flavipes* (two-tailed; $t_s=2.78$, $df=22$, $p=0.011$). Similarly, the proportion of full-sibling pairs, as assessed by Kinship, was significantly higher among *R. flavipes* tandem-running pairs (17 of 65) than among those of *R. virginicus* (8 of 156) (G test; $G_1=18.2$, $p<0.001$). Population-wide Kinship analyses indicated that *R. flavipes* had fewer opportunities to mate with non-nestmates than did *R. virginicus*. Individuals of the former species were significantly more likely (13.2% of 2,785 pairwise comparisons) to be full siblings with others sampled in the same population than were individuals of *R. virginicus* (2.3% of 17,101 pairwise comparisons) (G test; $G_1=526$, $p<0.001$).

In *R. virginicus*, we found no difference in relatedness between tandem-running pairs and those heading established colonies; both were about 0.05 (Table 2) (two-tailed; $t_s=0.11$, $df=8$, $p=0.46$). Analysis with Kinship 1.3.1 also did not reveal a difference between relatedness patterns in tandem-running pairs and those heading established colonies. The proportion of full-sibling pairs was low in both the tandem pairs (8 of 156) and the established pairs (0 of 16), and these proportions did not differ significantly from one another (Fisher's two-tailed exact test; $p=1.0$).

In *R. flavipes*, however, relatedness was significantly higher within tandem-running pairs ($r=0.130$) than within pairs heading established simple-family colonies ($r=0.011$) (two-tailed; $t_s=5.65$, $df=15$, $p<0.0001$). A comparison of the distributions of individual relatedness estimates, combined with the results of Kinship analysis, revealed a

Table 2 F statistics \pm SE and relatedness (r) for *R. virginicus* and *R. flavipes* reproductives (95% confidence interval in parentheses)

Group	F_{IS}	F_{ST}	r
<i>R. virginicus</i> primaries			
Mature colony pairs			0.053 \pm 0.059 (–0.087 to 0.192)
Tandem-running pairs			0.059 \pm 0.016 (0.025 to 0.093)
All termites	0.061 \pm 0.016 (0.029 to 0.091)	0.008 \pm 0.002 (0.004–0.012)	
Males only	0.018 \pm 0.022 (–0.024 to 0.057)	0.005 \pm 0.002 (0.000–0.009)	
Females only	0.106 \pm 0.019 (0.069 to 0.142)	0.010 \pm 0.003 (0.005–0.015)	
<i>R. flavipes</i> primaries			
Mature colony pairs			0.011 \pm 0.007 (–0.005 to 0.027)
Tandem-running pairs			0.130 \pm 0.020 (0.087 to 0.172)
All termites	0.102 \pm 0.056 (0.019 to 0.216)	0.034 \pm 0.007 (0.022–0.047)	
Males only	0.107 \pm 0.065 (0.010 to 0.240)	0.024 \pm 0.005 (0.014–0.034)	
Females only	0.099 \pm 0.051 (0.017 to 0.201)	0.037 \pm 0.010 (0.020–0.058)	

noteworthy difference between these two classes of *R. flavipes* primary reproductives. The distribution of relatedness values among tandem-running pairs deviated significantly from normal (Shapiro–Wilk W test, $p=0.043$; Fig. 1a) and was skewed to the right (skew=0.38). In contrast, relatedness values for pairs heading established colonies did not differ from normal distribution (Shapiro–Wilk W test, $p=0.79$; Fig. 1b). These differences appear to result from a significantly larger fraction of newly formed tandem pairs that consisted of full siblings (17 of 65 pairs; Fig. 1a) compared to those pairs heading established colonies (11 of 243; Fig. 1b) (G test; $G_1=23.4$, $p<0.0001$).

Several lines of evidence indicate that our comparison across populations is legitimate. First, relatedness patterns within established simple-family colonies show little variation across the six populations into which our comparative data could be grouped. The relatedness of established primary reproductives in these populations ranged from $-0.023(\pm 0.050)$ to $0.057(\pm 0.058)$, and none of these

estimates was significantly different from one another (for the two most extreme values: two-tailed; $t_s=1.04$, $df=11$, $p=0.31$). Furthermore, the relatedness of established primary reproductives did not appear to be affected by habitat type or colony density. Colonies collected from the immediate vicinity of human structures were still headed by unrelated reproductives ($n=144$, $r=0.004\pm 0.035$), and the density of established colonies within these properties had no significant effect on the proportion of these colonies headed by putative full siblings (comparing densely populated properties with ten or more colonies to those with fewer than ten; G test, $G_1=0.01$, $p=0.91$; this result did not change using other thresholds of colony density). Another line of evidence further indicates that we have not, in fact, stumbled upon an unusual population in which relatedness is high within established simple-family colonies. Preliminary data from a small number of colonies ($n=9$) from the current study site indicate that simple-family colonies outnumber those with three or more closely related reproductives by a factor of roughly two to one. As one would expect for a population with this family structure (Vargo 2003a,b; DeHeer and Vargo 2004), the newly produced kings and queens show only moderate levels of inbreeding ($F_{IS}=0.10$). If new colonies were commonly initiated by full siblings in this population, not only would their offspring be more inbred (expected $F_{IS}=0.33$ for full-sibling pairs; see Thorne et al. 1999), but colonies headed by replacement reproductives would often be inbred for an additional generation and would thus produce offspring with an even higher F_{IS} —combined, these would lead to a considerably higher F_{IS} than we, in fact, observed.

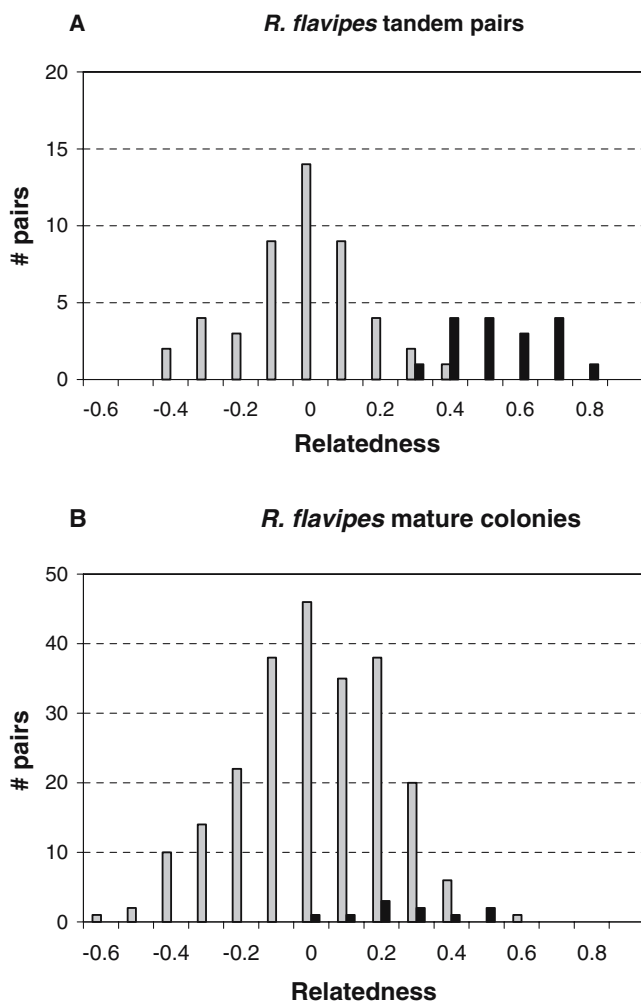


Fig. 1 The distribution of point estimates of relatedness between male and female *R. flavipes* reproductives within **a** tandem-running pairs and **b** pairs heading established simple-family colonies. The *black bars* represent pairs that were determined to be nestmates according to the Kinship program (see “Methods”), while the *grey bars* represent putatively non-nestmate pairs

Discussion

Newly formed tandem-running pairs in both *R. flavipes* and *R. virginicus* were, on average, significantly related to one another. This was largely the result of tandem-running pairs sometimes forming between nestmates, and the proportion of such nestmate pairs was substantially higher in *R. flavipes* (26.1%) than in *R. virginicus* (5.1%). The greater likelihood that *R. flavipes* paired with relatives correlated with between-species differences in their inferred dispersal ability. These differences are suggested by a suite of observations made during dispersal flights (see Table 1) and are confirmed by significantly higher estimates of and F_{ST} among recently flown *R. flavipes*.

A striking pattern from this study was that relatedness patterns within newly formed pairs in *R. flavipes* were substantially different from those between pairs of primary reproductives heading established colonies in this species (Fig. 1a,b), both in terms of relatedness estimates and the proportion of pairs formed between nestmate males and females. In contrast, *R. virginicus* tandem-running pairs exhibited patterns of genetic relatedness indistinguishable from single pairs of reproductives heading established colonies. The observed disparity in relatedness patterns for colony-founding pairs of *R. flavipes* and those within established colonies could arise from one of four causes.

First, because the current study occurred in a location different from that of the six populations analyzed from previous studies, it may be that there is spatial variation in relatedness among primary reproductives heading established colonies. However, the bulk of the evidence indicates that such dramatic variation across populations does not occur over the spatial scales studied here. Moreover, we did not detect any predictable variation across these six populations based upon the density of established colonies or the habitat in which they were collected (urban or forested areas). Lastly, the inbreeding coefficient reported from the current study is well within the range of variation described in these other studies despite the variety of habitats and spatial scales over which they were performed (e.g., Vargo 2003a,b; DeHeer and Vargo 2004), indicating that established colonies in the current study population differed little in their genetic organization from those within previously studied locations.

A second possible explanation for this disparity in relatedness patterns is that we have sampled an unusually sparse population of *R. flavipes* and that large distances between colonies have resulted in an abnormally high fraction of tandem pairs formed between nestmates. Although we agree that such variation in colony density would have a significant effect on pairing patterns, observations and genetic data do not support this hypothesis for the current study population. Even though established colonies are difficult to trap or excavate from this site, we observed numerous colonies emitting alates on days of dispersal flights, often within 5–15 m of one another (as is the case in nearby populations, Vargo 2003b; DeHeer and Vargo 2004). Allelic diversity in *R. flavipes* (10.4 alleles per locus, with two loci each having 24 alleles) was also consistent with that reported from nearby populations in which we have reported a high density of colonies (Vargo 2003b; DeHeer and Vargo 2004). Thus, it is likely that the density in the present study population was similar to that in other nearby populations and that the observed fraction of closely related tandem-running pairs is therefore representative of the populations in this geographic region.

The remaining two explanations for this excess of related tandem-running pairs are complementary hypotheses, which invoke either inbreeding avoidance or ID within *R. flavipes*. Inbreeding avoidance would be implicated if a large fraction of termites switched to unrelated mates subsequent to our sampling, if given the opportunity to do so. Such inbreeding avoidance has, in fact, been described during pair formation within *Z. nevadensis* (Shellman-Reeve 2001). Although we agree that such mate switching would have substantial benefits for both males and females, our field observations suggest that this was not a widespread occurrence in this population. We did not observe tandem-running pairs separating during the course of our collections, and we collected *R. flavipes* pairs until such time that they could no longer be found on the surface, suggesting that little subsequent above-ground mate choice was possible in these populations. Nevertheless, even if inbreeding avoidance occurred, it would provide indirect support for the existence of past or present ID. This latter process is, in fact,

our fourth explanation for the disappearance of these full-sibling pairs heading established *R. flavipes* colonies. We suggest that the closely related pairs of primary reproductives fail to initiate colonies during the critical colony-founding stage more often than do colonies headed by unrelated pairs. These results suggest that ID, either directly through selection or indirectly through inbreeding avoidance, plays an important role in the breeding system of at least this one species of termite.

Our test of ID within *R. virginicus* failed to detect any signature of selection against inbreeding pairs, although we do note that this test did not have high statistical power. Results of similar studies on other social species parallel those of the current study because they have also yielded mixed evidence for ID. Similarly, some other studies (Rosengaus and Traniello 1993; Bilde et al. 2005) have found only very minor costs or even a net benefit to inbreeding within inbred social or subsocial species, while others have described results suggesting that inbreeding may have a significant net cost to fitness (Shellman-Reeve 2001; Fei and Henderson 2003; Husseneder et al. in press). These mixed results both within and between studies may very well indicate that ID itself is highly variable across these taxa, just as it is among selfing plants (Husband and Schemske 1996; Byers and Waller 1999). The existence of significant ID could dramatically alter the potential benefits of inbreeding when considering the evolution of sociality in these taxa, and it could also be an important force in the evolution of breeding systems within inbreeding social organisms. Variation in ID could provide a framework to help explain variations in levels of inbreeding within and between species of termites, which appear to be considerable (cf. Reilly 1987; Thompson and Hebert 1998). A particularly interesting question is whether inbreeding has fitness effects for established colonies headed by highly related replacement reproductives. The hypothesis that there are fitness consequences for inbreeding within established colonies is at least circumstantially supported by patterns of inbreeding within the six nearby populations of *R. flavipes*. These populations show surprisingly low levels of inbreeding, considering the view that replacement reproductives should permit colonies to become very long-lived. However, not only are such colonies inbred in the minority (25%), but such colonies are inbred for very few generations (Vargo 2003a,b; DeHeer and Vargo 2004; Vargo unpublished data). A challenge for future works is to determine whether or not inbreeding has a detrimental effect within established colonies of these and other species, while taking care to avoid the experimental pitfalls associated with the measurement of ID (Hedrick and Kalinowski 2000; Keller and Waller 2002).

Our measures of genetic structure above the level of tandem pairs were consistent with our assessment of the probability that recently flown sexuals of each species will pair with a nestmate during tandem running. Differentiation (F_{ST}) among these populations was significantly greater than zero in both species at scales of about 100 m, and this differentiation was significantly higher in *R. flavipes* (0.034) than in *R. virginicus* (0.008). This was also

consistent with our observation that winged sexuals of the former species emerged from locations flushed to the ground, while those of *R. virginicus* usually emerged from treetop level. Furthermore, even though only a few studies have described the flight behavior of *R. virginicus* in detail, it may be significant that a report from an airborne insects survey has found this species flying at altitudes of up to 900 m (Snyder 1935). Some caution is necessary in the interpretation of F statistics generated with highly variable microsatellite markers (Bossart and Prowell 1998; Hedrick 1999). In particular, levels of differentiation are typically dependent on the levels of genetic variability, in such a way that highly variable markers will tend to yield lower estimates of F_{ST} than less variable markers under identical levels of migration. This trend, however, makes our conclusions conservative, since *R. flavipes* has considerably higher differentiation in spite of having greater allelic variability (a mean of 10.4 alleles per variable locus vs 6.1 in *R. virginicus*). To explore this idea further, we calculated the maximum possible F_{ST} for each species based on the numbers of alleles at each locus (maximizing differentiation such that each allele occurred in only one population and calculating F_{ST} using Formula 1a from Hedrick 1999). As one might expect, the maximum value of F_{ST} was lower in *R. flavipes* than in *R. virginicus* (0.334 vs 0.555). Differentiation was therefore about 10.2% of its maximum expected value in the former species and was 1.4% of its maximum expected value in the latter; thus, we suggest that these differences between species are biologically as well as statistically significant. Thus, based on differentiation among groups of recently flown alates, *R. flavipes* were much more likely to encounter close relatives than were *R. virginicus*.

Inbreeding coefficients (F_{IS}), the indirect measures of relatedness among parents of these new reproductives, were modest in magnitude but significantly greater than zero in both *R. flavipes* (0.102) and *R. virginicus* (0.061). This observation is consistent with genetic studies of nearly 300 established colonies found within 20-km radius of the current study. These studies indicate that colonies in both species are mostly headed by unrelated reproductives (presumably primaries), while a smaller fraction (25% in *R. flavipes*) is headed by secondary reproductives, which are close relatives to one another (Vargo 2003a,b; DeHeer and Vargo 2004; Vargo unpublished data). Curiously, alate inbreeding coefficients in *R. virginicus* showed marked sexual dimorphism, with females ($F_{IS}=0.106$) being significantly more inbred than males ($F_{IS}=0.018$). This suggests that more inbred colonies produce a larger proportion of females in these populations, but we have no data to determine whether or not this is a direct effect of inbreeding or an indirect effect of some colony-level correlate of inbreeding (e.g., colony size, number of reproductives).

The population genetics of social insects are seldom studied using reproductive castes, but instead tend to focus on omnipresent workers. For some questions of colony genetic structure (e.g., nestmate relatedness), sterile castes are, in fact, the preferred source material, but in order to

address other questions, researchers are sometimes obliged to assay the genotypes of reproductive individuals (e.g., sperm utilization, Chapuisat 1998; queen–queen relatedness, Rüppeell et al. 2002; sex-biased dispersal tendencies, Sundström et al. 2003). Even though one must have good timing or increased sampling effort in order to obtain reproductive castes, these studies demonstrate that this approach can yield novel insights into reproductive biology, which have gone unnoticed via the use of more conventional approaches. Likewise, we show in the current study that, at least in one population, recently flown *R. flavipes* often pair with nestmates immediately after dispersal flights—a conclusion not suggested by the study of the breeding system within established colonies. Although this finding lends credence to the often cited observation that many termites are very weak fliers, the disparity in inferred mating strategies strongly suggests that tolerance for inbreeding is not a universal characteristic of termite breeding systems.

Acknowledgement Tom Juba provided assistance in the field and in the laboratory. To the best of our knowledge, we performed this work in accordance with state and federal laws regulating scientific research.

References

- Avilés L (1997) Causes and consequences of cooperation and permanent-sociality in spiders. In: Crespi BJ, Choe J (eds) The evolution of social behavior in insects and arachnids. Cambridge University Press, Cambridge UK, pp 476–498
- Barrett SCH (1998) The evolution of mating strategies in flowering plants. *Trends Plant Sci* 3:335–341
- Barrett SCH, Harder LD (1996) Ecology and evolution of plant mating. *Trends Ecol Evol* 11:73–79
- Bartz SH (1979) Evolution of eusociality in termites. *Proc Natl Acad Sci U S A* 76:5764–5768 (correction, *PNAS* 77:3070)
- Bilde T, Lubin Y, Smith DR, Schneider JM, Maklakov AA (2005) The transition to social inbred mating systems in spiders: role of inbreeding tolerance in a subsocial predecessor. *Evolution* 59:160–174
- Bossart JL, Prowell DP (1998) Genetic estimates of population structure and gene flow: limitations, lessons and new directions. *Trends Ecol Evol* 13:202–206
- Braude S (2000) Dispersal and new colony foundation in wild naked mole-rats: evidence against inbreeding as the system of mating. *Behav Ecol* 11:7–12
- Bulmer MS, Adams ES, Traniello JFA (2001) Variation in colony structure in the subterranean termite *Reticulitermes flavipes*. *Behav Ecol Sociobiol* 49:236–243
- Byers DL, Waller DM (1999) Do plant populations purge their genetic load? Effects of population size and mating history on inbreeding depression. *Annu Rev Ecol Syst* 30:479–513
- Chapuisat M (1998) Mating frequency of ant queens with alternative dispersal strategies, as revealed by microsatellite analysis of sperm. *Mol Ecol* 7:1097–1105
- Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. *Annu Rev Ecol Syst* 18:237–268
- Ciszek D (2000) New colony formation in the “highly inbred” eusocial naked mole-rat: outbreeding is preferred. *Behav Ecol* 11:1–6
- Cook JM, Crozier RH (1995) Sex determination and population biology in the Hymenoptera. *Trends Ecol Evol* 10:281–286

- DeHeer CJ, Vargo EL (2004) Colony genetic organization and colony fusion in the termite *Reticulitermes flavipes* as revealed by foraging patterns over time and space. *Mol Ecol* 13:431–441
- DeHeer CJ, Kutnik M, Bagnères A-G, Vargo EL (2005) The breeding system and population structure of the termite *Reticulitermes grassei* in southwestern France. *Heredity* 95:408–415
- Dronnet S, Bagnères A-G, Juba TR, Vargo EL (2004) Polymorphic microsatellite loci in the European subterranean termite, *Reticulitermes santonensis* Feytaud. *Mol Ecol Notes* 4:127–129
- Dronnet S, Chapuisat M, Vargo EL, Lohou C, Bagnères A-G (2005) Genetic analysis of the breeding system of an invasive subterranean termite, *Reticulitermes santonensis*, in urban and natural habitats. *Mol Ecol* 14:1311–1320
- Fei HX, Henderson G (2003) Comparative study of incipient colony development in the Formosan subterranean termite *Coptotermes formosanus* Shiraki (Isoptera, Rhinotermitidae). *Insect Soc* 50:201–297
- Hamilton WD (1972) Altruism and related phenomena, mainly in social insects. *Annu Rev Ecol Syst* 3:193–232
- Hedrick PW (1999) Perspective: highly variable loci and their interpretation in evolution and conservation. *Evolution* 53:313–318
- Hedrick PW, Kalinowski ST (2000) Inbreeding depression in conservation biology. *Annu Rev Ecol Syst* 31:139–162
- Henter HJ (2003) Inbreeding depression and haplodiploidy: experimental measures in a parasitoid and comparisons across diploid and haplodiploid insect taxa. *Evolution* 57:1793–1803
- Howard RW, Jones SC, Mauldin JK, Beal RH (1982) Abundance, distribution, and colony size estimates for *Reticulitermes* spp (Isoptera: Rhinotermitidae) in southern Mississippi. *Environ Entomol* 11:1290–1293
- Husband BC, Schemske DW (1996) Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* 50:54–70
- Husseneder C, Messenger MT, Su N-Y, Grace JK, Vargo EL (2005) Colony social organization and population genetic structure of an introduced population of the Formosan Subterranean Termite from New Orleans, Louisiana, USA. *J Econ Entomol* (in press)
- Jarvis JUM, O’Riain MJ, Bennett NC, Sherman PW (1994) Mammalian eusociality: a family affair. *Trends Ecol Evol* 9:47–51
- Jones SC, La Fage JP, Howard RW (1988) Isopteran sex ratios: phylogenetic trends. *Sociobiology* 14:89–156
- Keller LF, Waller DM (2002) Inbreeding effects in wild populations. *Trends Ecol Evol* 17:230–241
- Michod RE (1980) Evolution of interactions in family-structured populations: mixed mating models. *Genetics* 96:275–296
- Myles TG (1999) Review of secondary reproduction in termites (Insecta: Isoptera) with comments on its role in termite ecology and social evolution. *Sociobiology* 33:1–87
- Nutting WL (1969) Flight and colony foundation. In: Krishna K, Weesner FM (eds) *Biology of termites*, vol 1. Academic, New York, pp 233–282
- Pearce MJ (1997) *Termites: biology and pest management*. CAB International, New York, NY pp 233–282
- Pusey A, Wolf M (1996) Inbreeding avoidance in animals. *Trends Ecol Evol* 11:201–206
- Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. *Evolution* 43:258–275
- Reilly LM (1987) Measurements of inbreeding and average relatedness in a termite population. *Am Nat* 130:339–349
- Rosengaus RB, Traniello JFA (1993) Disease risk as a cost of outbreeding in the termite *Zootermopsis angusticollis*. *Proc Natl Acad Sci U S A* 90:6641–6645
- Ross KG (2001) Molecular ecology of social behaviour: analyses of breeding systems and genetic structure. *Mol Ecol* 10:265–284
- Rüppell O, Heinze J, Hölldobler B (2002) Intracolony patterns of reproduction in the queen-size dimorphic ant *Leptothorax rugatulus*. *Behav Ecol* 13:239–247
- Shellman-Reeve JS (1996) Operational sex ratios and lipid reserves in the dampwood termite *Zootermopsis nevadensis* (Hagen) (Isoptera: Termopsidae). *J Kans Entomol Soc* 69:139–146
- Shellman-Reeve JS (2001) Genetic relatedness and partner preference in a monogamous, wood-dwelling termite. *Anim Behav* 61:869–876
- Snyder TE (1935) *Our enemy the termite*. Comstock, Ithaca, NY
- Sokal RR, Rohlf FJ (1981) *Biometry*. Freeman, New York NY
- Sundström L, Keller L, Chapuisat M (2003) Inbreeding and sex-biased gene flow in the ant *Formica exsecta*. *Evolution* 57:1552–1561
- Thompson GJ, Hebert PDN (1998) Population genetic structure of the Neotropical termite *Nasutitermes nigriceps* (Isoptera: Termitidae). *Heredity* 80:48–55
- Thorne BL, Traniello JFA, Adams ES, Bulmer MS (1999) Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera Rhinotermitidae): a review of the evidence from behavioral, ecological, and genetic studies. *Ethol Ecol Evol* 11:149–169
- Uyenoyama MK (1984) Inbreeding and the evolution of altruism under kin selection: effects of relatedness and group structure. *Evolution* 38:778–795
- Vargo EL (2000) Polymorphism at trinucleotide microsatellite loci in the subterranean termite *Reticulitermes flavipes*. *Mol Ecol* 9:817–829
- Vargo EL (2003a) Genetic structure of *Reticulitermes flavipes* and *R. virginicus* (Isoptera: Rhinotermitidae) colonies in an urban habitat and tracking of colonies following treatment with hexaflumuron bait. *Environ Entomol* 32:1271–1282
- Vargo EL (2003b) Hierarchical analysis of colony and population genetic structure in the eastern subterranean termite, *Reticulitermes flavipes*, using two classes of molecular markers. *Evolution* 57:2805–2818
- Wang S, Hard JJ, Utter F (2002) Salmonid inbreeding: a review. *Rev Fish Biol Fish* 11:301–319
- Waser PM, Austad SM, Keane B (1986) When should animals tolerate inbreeding? *Am Nat* 128:529–537
- Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution* 38:1358–1370