

# Comparative Study of Breeding Systems of Sympatric Subterranean Termites (*Reticulitermes flavipes* and *R. hageni*) in Central North Carolina Using Two Classes of Molecular Genetic Markers

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**ABSTRACT** We used microsatellite and mtDNA markers to compare colony and population genetics of two sympatric species of subterranean termites, *Reticulitermes flavipes* (Kollar) ( $n = 25$  colonies) and *R. hageni* Banks ( $n = 16$  colonies), in two sites located 27 km apart in the Piedmont of central North Carolina. Colony breeding structure was inferred by examination of microsatellite genotypes of workers within colonies and by estimates of nestmate relatedness and hierarchical  $F$ -statistics. Similar to previous results on this species, nearly one-half of the *R. flavipes* colonies were simple families mainly headed by outbred primary (alate-derived) reproductives, about one-half were comprised of extended (inbred) families inferred to be headed by low numbers of neotenic (non alate-derived secondary reproductives) descended from the original primary pair, and two colonies contained the offspring of multiple reproductives. About two-thirds of the *R. hageni* colonies were comprised of simple families largely headed by related reproductives, and about one-third consisted of extended families headed by low numbers of neotenic. *R. hageni* differed from *R. flavipes* in having significant isolation by distance at one site as well as significant differentiation between sites at both the microsatellite and mtDNA markers. We conclude that dispersal in *R. hageni* is more limited resulting in higher levels of inbreeding within colonies and greater degrees of population genetic structure at small and large spatial scales than in sympatric populations of *R. flavipes*. These results indicate that closely related species of subterranean termites occurring in the same habitat can differ in their breeding systems with important consequences for higher level genetic structure.

**KEY WORDS** *Reticulitermes flavipes*, *Reticulitermes hageni*, *Reticulitermes virginicus*, microsatellites, mitochondrial DNA

TERMITES (ISOPTERA) ARE A LARGE and ecologically important group of social insects. Following the basic ground plan of highly cooperative insect societies, termite colonies consist primarily of nonreproductive workers who labor in support of relatively few reproductively specialized individuals. Within this general reproductive theme, however, termites exhibit considerable variation in colony breeding structure, primarily concerning the numbers of reproductives within colonies and the degree of relatedness among them. Both of these variables largely depend on whether colonies are simple families headed by monogamous pairs of adult-form (primary) reproductives or whether colonies are extended families containing numerous neotenic reproductives, nonwinged reproductive forms who develop within established colonies as precocial breeders from either nymphs or workers (Shellman-Reeve 1997, Myles 1999). In general, primary reproductives disperse through mating flights to found new colonies, whereas neotenic remain in their natal colony where they inbreed with

each other. Thus, the colony breeding system and associated dispersal behaviors can strongly affect colony and population genetic structure: simple family colonies headed by dispersive primary reproductives promote gene flow and outbreeding, whereas extended family colonies headed by nondispersive neotenic lead to inbreeding and increased genetic contrasts among colonies. There is considerable variation among termite taxa in their tendency to form neotenic-headed colonies, and this variation is related to ecological conditions and behavioral traits. Specifically, frequent production of neotenic seems to be an ancestral trait associated with the formation of temporary nest sites located in or close to relatively unstable food sources (Shellman-Reeve 1997, Myles 1999).

Subterranean termites (Rhinotermitidae), in particular, have exceptionally plastic breeding systems and dispersal behavior, with a strong tendency to produce neotenic and to form extended family colonies. The Holarctic genus *Reticulitermes* is the best studied group of subterranean termites with respect to breeding structure, and work on this genus has been

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accelerating, largely because of the availability of sensitive molecular markers (Clément 1981, 1984, Reilly 1987, Jenkins et al. 1999, Bulmer et al. 2001, Clément et al. 2001, Bulmer and Traniello 2002a, b, Vargo 2003a, b, DeHeer and Vargo 2004, Dronnet et al. 2004, 2005, DeHeer et al. 2005). These studies show variable breeding structures and suggest that local ecological conditions are important in shaping colony reproductive structure. In the North American species, *R. flavipes* (Kollar), Bulmer et al. (2001) reported differences in breeding structure between two Massachusetts sites located 0.5 km apart, based on allozyme and mitochondrial DNA (mtDNA) markers. One site with rocky and poorly drained soil consisted of a mixture of extended families (60%) and simple families (40%), all with limited foraging ranges, whereas the other site containing more porous soil had equal numbers of spatially expansive extended families and mixed family colonies. Three recent studies of *R. flavipes* from central North Carolina using microsatellite markers give consistent results concerning colony breeding structure in this geographic region (Vargo 2003a, b, DeHeer and Vargo 2004): some 75% of the 126 colonies studied were simple families, 24% were neotenic-headed extended families descended from simple families, and a single colony contained genetically diverse individuals originating from the fusion of two distinct colonies. Thus, *R. flavipes* shows variation in breeding structure at both small and large spatial scales, presumably in response to ecological conditions.

Although *R. flavipes* is only one of six recognized species of *Reticulitermes* in the United States, and there are probably several more unrecognized species (Austin et al. 2002, Page et al. 2002, Copren et al. 2005), almost nothing is known concerning the breeding system of the other species. Over most of its range in the eastern United States, *R. flavipes* is sympatric with two congeners, *R. hageni* Banks and *R. virginicus* (Banks) (Snyder 1954, Austin et al. 2002). Five colonies of the latter species in North Carolina have been analyzed using microsatellite markers (Vargo 2003b, DeHeer and Vargo 2004); four of these were simple families and one was an extended family. Only a single *R. hageni* colony, also from North Carolina, has been genetically characterized, and it was a simple family (DeHeer and Vargo 2004).

To better understand the causes and consequences of variation in breeding system within and among *Reticulitermes* spp., we undertook a comparative study of colony and population genetic structure of *R. flavipes* and *R. hageni* in two forests in central North Carolina. Although *R. flavipes* is reasonably well studied in this area (Vargo 2003a, b, DeHeer and Vargo 2004), we included it in this study to control for variation in breeding structure caused by local ecological conditions or temporal differences. We used both microsatellite markers (Vargo 2000) and mtDNA markers to infer the breeding structure of colonies and to assess relative gene flow within and among popula-

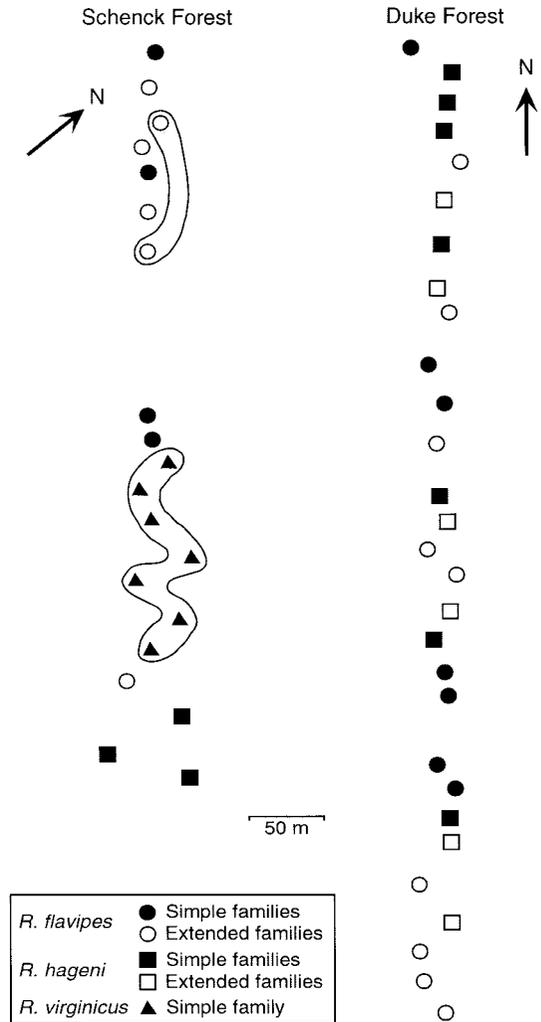


Fig. 1. Relative locations of *R. flavipes*, *R. hageni*, and *R. virginicus* colonies sampled in the two transects.

## Materials and Methods

### Sample Collection

In May and June 2000, *Reticulitermes* spp. termites were sampled from two undisturbed forest habitats: Schenck Forest, a facility owned and managed by North Carolina State University located in western Wake County, NC, and Duke Forest, belonging to Duke University and located in central Durham County, NC. These sites, which consisted of mixed hardwoods and loblolly pine (*Pinus taeda* L.) >50 yr old, are located  $\approx 27$  km apart. Samples of at least 50 workers, some soldiers, and occasionally alates were collected from natural wood debris along approximately linear transects in each forest with a minimum of 15 m between collection points (Fig. 1). The distance between collection points was measured and recorded. Samples were taken from 20 collection points in Schenck Forest and from 29 collection points

in Duke Forest. Originally, this project was meant to be a study of *R. virginicus*, a species whose worker and soldier body sizes are intermediate between the other two *Reticulitermes* spp. occurring locally, the larger and more common *R. flavipes* and the smaller *R. hageni*. When samples were encountered that appeared to be too large for *R. virginicus*, they were not collected. Any samples that appeared too small to be *R. flavipes* were collected. However, on closer inspection, and especially after use of molecular markers, we determined that the samples consisted of a mixture of all three *Reticulitermes* spp., primarily *R. flavipes* and *R. hageni*. Thus, samples of the larger *R. flavipes* were biased toward colonies with small workers, whereas there was no obvious bias in the *R. hageni* samples.

Immediately on collection, samples were placed into vials containing 95% ethanol. The day after collection, the ethanol was replaced, and any dirt or debris was removed from the vial. The vials were stored at  $-20^{\circ}\text{C}$  until DNA extraction.

### Species Identification

Soldiers in each sample were examined morphologically for species identification using the keys of Scheffrahn and Su (1994) and Hostettler et al. (1995). According to these keys, *R. flavipes* generally has a soldier pronotal width  $\geq 0.85$  mm and the point of the left mandible curves inward at  $70-90^{\circ}$ , that of *R. virginicus* usually measures 0.71–0.80 mm with the point of the left mandible curves inward at  $70-90^{\circ}$ , whereas that of *R. hageni* is generally  $< 0.70$  mm and the point of the left mandible curves inward at  $45^{\circ}$ . From one to three soldiers per sample were examined for both pronotal width and mandible curvature. We determined that identification based on morphological measurements proved unreliable; many specimens that keyed to *R. virginicus* by these criteria were later reclassified based on sequence data from the *cytochrome oxidase II* (COII) gene as either *R. flavipes* or *R. hageni* (Szalanski et al. 2003). Voucher specimens have been deposited in the North Carolina State University Insect Collection.

### Genotyping

Individual whole bodies of termite workers were pulverized in liquid nitrogen, and genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). At least 20 workers from each collection point were genotyped at eight trinucleotide microsatellite loci following the methods of Vargo (2000), in which the polymerase chain reaction (PCR) products are labeled by the addition of a fluorescent labeled primer to the reaction. The PCR products were separated in 6.5% polyacrylamide sequencing gels run on a Li-Cor 4000 or 4200 automated DNA sequencer. Locus *Rf 21-1* was amplified separately, but the other seven primer pairs were multiplexed in the following combinations: (*Rf 1-3* and *Rf 5-10*), (*Rf 6-1*, *Rf 11-2* and *Rf 15-2*), (*Rf 11-1* and *Rf 24-2*). Gel images were scored using GeneImagIR v. 3.56 (Scanalytics, Inc.,

Fairfax, VA) to obtain individual genotypes. Mendelian inheritance of all loci was shown previously for *R. flavipes* by Vargo (2003a).

The COII gene of mtDNA was sequenced using the primers A-t Leu and B-t Lys (Liu and Beckenbach 1992). In most cases, two individuals per collection point were sequenced, but more individuals were sequenced in the two collection points containing mixed family groups (see Results). Reaction volumes were  $50.0 \mu\text{l}$ , containing  $\approx 20$  ng ( $4 \mu\text{l}$ ) of genomic DNA,  $1 \times$  PCR reaction buffer, 2 mM  $\text{MgCl}_2$ , 0.25 mM dNTPs, 0.06 U Biolase *Taq* polymerase (Biolone, Canton, MA), and 1 pmol of each primer. PCR was performed on a PTC-100 thermal cycler (MJ Research, Littleton, MA) using the following program: initial denaturation at  $94^{\circ}\text{C}$  (2 min) followed by 35 cycles of 94 (1 min), 50 (1 min) and 70 (2 min), with a final extension step at  $70^{\circ}\text{C}$  (5 min).

PCR products were either gel purified or cleaned up using the QIAquick PCR Purification kit (Qiagen) before amplification using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Both forward and reverse reactions were performed using  $1 \times$  ABI reaction mixture (ABI Prism DRhodamine Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems) and 0.3 pmol of primer A-t Leu or B-t Lys. The dye terminators were removed from the sequencing reaction mixture by passing the solution through CentriSep columns (Princeton Separations, Adelphia, NJ) according to the manufacturer's instructions. Products were dried on a speed-vac and submitted to the North Carolina State University Sequencing Facility, where the samples were run on an ABI 377 automated sequencer. Each template was sequenced in both directions. Sequence editing and alignment were performed using the software program Vector NTI Suite (Informax, Frederick, MD).

### Genetic Data Analysis

**Tests for Hardy-Weinberg Equilibrium and Linkage Disequilibrium.** Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were analyzed by means of exact tests using the program Genetic Data Analysis v.1.1 (Lewis and Zaykin 2000) with 3,200 iterations. Because genotypes within colonies were not independent due to the close family relationships among colony mates (see Results), only a single individual per colony was used for these tests. We used a resampling procedure in which one individual from each colony was selected at random for a total of 20 replications.

**Colony Affiliations.** Colony associations for the termites from each collection site were made based on microsatellite genotypes and mtDNA haplotypes. For each of the two sites, genotypes of workers from each collection point were compared with those of all other collection points of the same species. First, we compared the number of private alleles, i.e., alleles unique to one group of workers, and the genotypes present at all microsatellite loci. Collection points were consid-

ered part of the same colony if they had all the same alleles present and a compatible set of genotypes at all microsatellite loci, and they had identical mtDNA haplotypes. In addition, we performed pairwise  $F_{ST}$ s among all conspecific pairs of collection points within each transect using the program FSTAT (Goudet 2001). The significance of the  $F_{ST}$ s was assessed by a permutation test performed by FSTAT using 1,000 iterations and the Bonferroni correction applied. Non-significant  $F_{ST}$ s between workers from different collection points were taken as additional evidence that collection points were part of the same colony. In practice, the high variability of the markers and the close family structure of colonies (see Results) made colony designations unambiguous.

**Classification of Colonies.** Following the terminology of Vargo (2003a, b), Vargo et al. (2003), and DeHeer and Vargo (2004), colonies were classified as simple families, extended families, or mixed families. Simple family colonies were those in which worker genotypes at all microsatellite loci were consistent with being the offspring of monogamous pairs of reproductives, and the observed frequencies of the genotypes did not differ significantly from the expected Mendelian ratios. Deviation of genotype frequencies was assessed by means of a  $G$ -test in which the locus specific  $G$ -values were summed to obtain an overall  $G$ -value for each colony. Colonies were considered extended families if they contained no more than four alleles per locus and had genotypes compatible with a single pair of reproductives at one or more loci (e.g., five or more genotypic classes or three classes of homozygotes), or they had genotypes that were compatible with simple families but their frequencies deviated significantly from expected ( $P < 0.05$ ,  $G$ -test). Extended family colonies presumably were headed by multiple neotenic reproductives by themselves or possibly together with one or both primary reproductives. Mixed families had more than four alleles at a locus indicating the presence of multiple same-sex reproductives originating from outside the colony.

**Colony and Population Genetic Structure.** Colony and population genetic structure were assessed at several levels. To determine kin structure within colonies, we estimated the average relatedness for workers from the microsatellite genotype frequencies using the program Relatedness v.5.0.8 (Queller and Goodnight 1989) with colonies weighted equally. SEMs were obtained by jackknifing over loci. For *R. hageni*, within-colony relatedness was corrected for genetic structure between sites using the Demes option, because there was significant differentiation between the two sites in this species (see Results). The significance of the coefficients was determined by comparing them to zero with a  $t$ -test. A two-sample approximate  $t$ -test assuming unequal variances (Sokal and Rohlf 1981) was used to determine whether two coefficients were significantly different.

Structure at the level of the colony, the transect, and site was assessed simultaneously by estimating  $F$ -statistics using the method of Weir and Cockerham (1984) as implemented in FSTAT. We followed the

notation of Thorne et al. (1999) and Bulmer et al. (2001) in which genetic variation is partitioned among the individual (I), colony (C), and total (T) components. Using this notation,  $F_{IT}$  is equivalent to the standard inbreeding coefficient  $F_{IS}$  in the absence of differentiation between transects,  $F_{CT}$  represents genetic differentiation among colonies and is similar to  $F_{ST}$ , and  $F_{IC}$  is the colony inbreeding coefficient. This last term has no analog in solitary organisms; it is especially sensitive to the numbers of reproductives and their mating patterns within social groups and is therefore highly informative in inferring colony breeding structure (Thorne et al. 1999). In addition, we estimated  $F_{ST}$ -values among transects within sites as well as the overall  $F_{ST}$  among sites using Genetic Data Analysis. SEs of the  $F$ -values obtained using FSTAT were generated by jackknifing over loci. Significance of the values was determined by means of a one-sample  $t$ -test; a two-sample approximate  $t$ -test assuming unequal variances (Sokal and Rohlf 1981) was used to determine significance when comparing  $F$ -values of two groups.  $F$ -statistics were estimated for all colonies together, as well as for simple family colonies and extended family colonies separately. For *R. hageni*, analyses of colony-level  $F$ -statistics were done separately for the two transects to eliminate effects caused by higher level structure between transects (see Results). These values were compared with values generated by the methods of Thorne et al. (1999) in computer simulations of possible breeding systems of *Reticulitermes* species. Genotypes of the reproductives in each simple family colony were inferred from worker genotypes.  $F$ -statistics and the average relatedness between the reproductives in these colonies were estimated from the reconstructed genotypes. In addition, we used the program Kinship v. 1.3.1 (Goodnight and Queller 1999) to determine the probability that the nestmate reproductives within each simple family were close relatives. This program uses maximum likelihood methods to determine whether pairs of individuals share a specified pedigree relationship. We tested whether the two reproductives within each simple family were putative full siblings by testing each pair against the average coefficient of relatedness among workers in each population and using zero as the null hypothesis. The statistical significance of the results was based on 10,000 permutations.

To ensure that the *COII* gene was not under strong selection, sequences were tested for conformance to the neutral mutation hypothesis by means of the  $D$  statistic of Tajima (1989) as implemented in the program DnaSP (Rozas and Rozas 1999, available at <http://www.ub.es/dnasp/>). We also calculated nucleotide diversity ( $\pi$ ), defined as the average number of pairwise nucleotide differences per site, and  $\theta$  ( $=2N_f\mu$ , where  $N_f$  is the effective population size of females and  $\mu$  is the mutation rate) for each species and each population using this program. Population genetic structure derived from the mtDNA sequence data were assessed at the level of the transect by estimating  $\Phi$ -statistics using the analysis of molecular variance method of Excoffier et al. (1992) as implemented in

the program ARLEQUIN (Raymond and Rousset 1995). This analysis was performed on the Euclidian square distances between pairs of haplotypes according to the method of Tajima and Nei (1984), and the significance probabilities for the  $\Phi$ -statistics were generated using permutation analysis on 10,000 randomly permuted distance matrices. The program Molecular Evolutionary Genetics Analysis v.3.1 (Kumar et al. 2004) was used to construct a neighbor-joining tree depicting the genetic relationships of the haplotypes from the pairwise Euclidian distances, and confidence levels for the nodes were obtained by bootstrapping 1,000 times. In addition, a minimum spanning tree was constructed using ARLEQUIN.

**Isolation by Distance Analysis.** Isolation by distance was assessed from the microsatellite genotypes by estimating the  $F_{CT}$  between workers from one colony and those in another colony for all pairs of conspecific colonies within each transect. The Pearson product correlation coefficient was computed for the pairwise  $F_{CT}$ -values and the physical distance for all colony pairs within each site. The significance of the correlation coefficients was assessed by means of a Mantel test with 1,000 replications as implemented in Genepop on the Web v. 3.1c (Raymond and Rousset 1995).

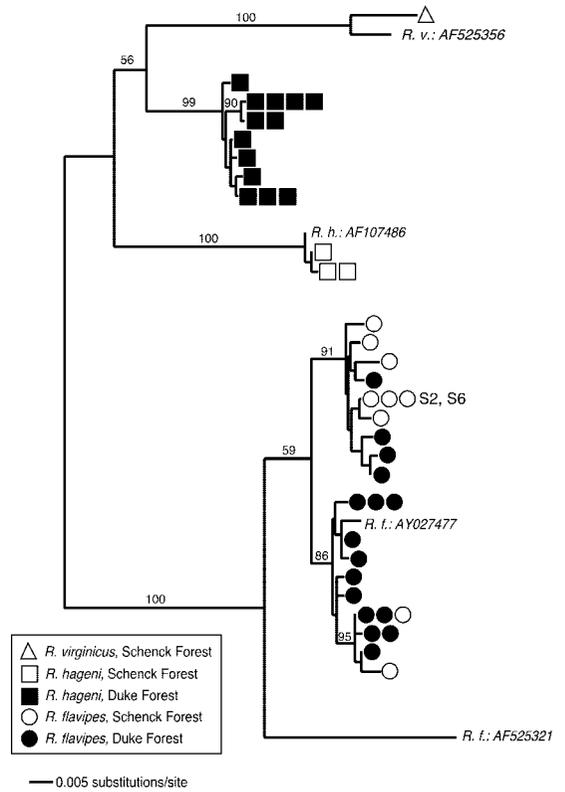
**Results**

**Species Identification**

Based on soldier pronotal width and mandible shape, samples from 9 of the 49 total collection points keyed to *R. hageni* (mean pronotal width  $\pm$  SD =  $0.67 \pm 0.03$ , range =  $0.64-0.72$ ,  $n = 15$ ), whereas 39 samples keyed to *R. virginicus* (mean =  $0.81 \pm 0.04$ , range =  $0.72-0.88$ ,  $n = 67$ ); 1 sample did not contain soldiers. However, in the course of examining sequence variation in the *COII* gene, we found that the samples separated into three groups, aligning themselves with sequences from GenBank for *R. flavipes*, *R. virginicus* or *R. hageni* (Fig. 2). Szalanski et al. (2003) also found that the *COII* gene provides a reliable means to distinguish among these three species. We used this grouping as the basis for our species identifications, yielding 26 samples of *R. flavipes* (10 from Schenck Forest and 16 from Duke Forest), 16 samples of *R. hageni* (3 from Schenck Forest and 13 from Duke Forest), and 7 samples of *R. virginicus*, all from Schenck Forest and all with identical haplotypes. The mean number of base pair differences among species was three to four times higher than within species (Table 1). The mean pronotal widths for soldiers of these three species in our sample were  $0.82 \pm 0.03$  for *R. flavipes* ( $n = 49$ ),  $0.78 \pm 0.02$  for *R. virginicus* ( $n = 12$ ), and  $0.71 \pm 0.06$  for *R. hageni* ( $n = 24$ ).

**Basic Genetic Data**

A total of 1,140 individuals was genotyped at eight microsatellite loci, including 680 *R. flavipes* workers, 320 *R. hageni* workers, and 140 *R. virginicus* workers. As shown in Table 2, there were 3-27 alleles per locus



**Fig. 2.** Neighbor-joining tree of *COII* haplotypes: length = 164; CI = 0.67, RI = 0.90. Sequences for each species from GenBank were included for comparison.

present in *R. flavipes*, whereas the *R. hageni* population was slightly less polymorphic with between 3 and 20 alleles per locus present. Based on mtDNA haplotype and microsatellite genotypes, we determined that all of the *R. virginicus* samples belonged to the same simple family colony. Because this species was represented by a single colony, we excluded it from further analysis and confined our comparison to *R. flavipes* and *R. hageni*.

For *R. flavipes*, locus *Rf 11-2* deviated significantly from HWE in 16 of the 20 resampled data sets, whereas none of the other loci showed significant deviation in more than two cases. Locus *Rf 11-2*, which also was found to deviate significantly from HWE in another study of nearby populations of *R. flavipes* (Vargo 2003a), was excluded from subsequent analyses. The mean number of alleles for the remaining seven loci was  $12.6 \pm 8.7$ . For *R. hageni*, five loci showed no

**Table 1.** Mean no. base pair substitutions in the *COII* gene (length = 680 bp) within and among *Reticulitermes* spp. from the study area

	<i>R. flavipes</i>	<i>R. virginicus</i>	<i>R. hageni</i>
<i>R. flavipes</i> ( $n = 19$ )	10.0		
<i>R. virginicus</i> ( $n = 1$ )	53.9	—	
<i>R. hageni</i> ( $n = 9$ )	42.1	34.1	14.8

**Table 2.** Allele numbers and frequency of most common allele at eight microsatellite loci for the *R. flavipes* and *R. hageni* study populations

Locus	<i>R. flavipes</i>		<i>R. hageni</i>	
	No. alleles	Freq. most common allele	No. alleles	Freq. most common allele
<i>Rf 1-3</i>	10	0.36	6	0.29
<i>Rf 5-10</i>	7	0.62	7	0.68
<i>Rf 6-1</i>	12	0.47	11	0.37
<i>Rf 11-1</i>	7	0.47	5	0.67
<i>Rf 11-2</i>	4	0.78	3	0.87
<i>Rf 15-2</i>	3	0.67	6	0.3
<i>Rf 21-1</i>	22	0.29	20	0.13
<i>Rf 24-2</i>	27	0.17	13	0.25
Mean $\pm$ SD	11.5 $\pm$ 8.6		8.9 $\pm$ 5.5	

significant deviation from HWE in any of the 20 resampled data sets. Three loci deviated significantly in some cases—*Rf 5-10* in six cases, *Rf 11-1* in five cases, and *Rf 6-1* in one case—but we did not consider these deviations sufficient to warrant exclusion of these loci. There was no evidence of LD in either species, because none of the 560 tests performed for each species (28 pairs of loci  $\times$  20 resampled data sets) were significant. Thus, all eight loci in *R. hageni* and seven in *R. flavipes* were considered independently assorting markers suitable for colony and population genetic analysis.

Of the 680 bp in the *COII* gene, there were 103 (15.1%) variable positions with 82 (12.1% of total) of these parsimony informative. In all cases, the individuals sampled from the same colony had identical haplotypes. In addition, we performed PCR-restriction fragment-length polymorphism analysis of the A+T-rich region of mtDNA on 20 individuals per colony as described by Vargo (2003a), and in every case, only a single haplotype was present in each colony. *R. flavipes* and *R. hageni* showed similar haplotype diversity (Table 3). The neighbor-joining tree of haplotypes is shown in Fig. 2. The *R. flavipes* GenBank sequences were placed in the largest branch. Colonies on this branch were considered to be *R. flavipes*. The *R. hageni* samples formed two branches, one containing the Schenck Forest haplotypes and the other possessing the Duke Forest haplotypes. Within *R. flavipes*, there were 18 haplotypes, 7 in Schenck Forest and 12 in Duke Forest, of which only 1 was shared in common (Table 3; Fig. 2). The frequency of the most common haplotype was 0.33, and there were 14 unique haplo-

types. In *R. hageni*, there were nine haplotypes overall, two in Schenck Forest and seven in Duke Forest. There were no haplotypes shared in common between locations. There were five unique haplotypes, and the frequency of the most common haplotype was 0.25 (Table 3; Fig. 3).

### Colony Designations

Figure 1 shows the relative locations of colonies of the different species in the two transects. *R. flavipes* was the most commonly collected species, accounting for 26 (53%) of the 49 samples collected and 25 (60%) of the 42 colonies represented (Table 4). Based on the following evidence, all of the *R. hageni* collection points were considered distinct colonies. There was an average of  $18.7 \pm 4.5$  (SD) private alleles (range = 10–30) between pairs of collection points and the mean  $F_{CT}$ s for all pairs of collection points within a transect was  $0.36 \pm 0.09$  (range = 0.18–0.60), and all of these were significant at  $P < 0.01$  (Bonferroni correction applied). Of all the *R. flavipes* samples, only two collection points separated by 82.6 m had identical alleles and genotypes present. The  $F_{CT}$  between these two points was not significantly different from zero ( $-0.01$ ,  $P > 0.77$ ), and the coefficient of relatedness between workers in the two samples was high ( $r = 0.425 \pm 0.15$ ) and did not differ significantly from the coefficient of relatedness among workers within each sample ( $r = 0.495$  and  $0.560$ ; both  $P \geq 0.27$ , approximate  $t$ -test). In addition, they had the same mtDNA haplotype. Therefore, these two collection points were considered part of the same colony. In contrast, all other pairs of collection points within a transect were clearly different from each other, with an average of  $20.4 \pm 4.6$  private alleles (range = 10–36) and mean pairwise  $F_{CT} = 0.26 \pm 0.06$  (range = 0.12–0.40) significant at  $P < 0.001$  (Bonferroni correction applied); these were therefore considered to be distinct colonies.

### Colony Genetic Structure

The family structure of the *R. flavipes* and *R. hageni* colonies is shown in Table 4. With two exceptions, *R. flavipes* colonies S2 and S6 from the Schenck Forest transect (Tables 5 and 6), all colonies had at most four alleles at a locus, suggesting that they were comprised of the descendants of a single pair of founding repro-

**Table 3.** Haplotype and nucleotide diversity for the *COII* gene in *R. flavipes* and *R. hageni*

	N	No. haplotypes	Haplotype diversity	No. variable sites	$\pi$	$\theta$	SD ( $\theta$ )
<i>R. flavipes</i>	25	18	0.967	34	0.0147	0.0145	0.0055
Duke Forest	16	12	0.958	27	0.0134	0.0132	0.0055
Schenck Forest	9	7	0.917	21	0.0146	0.0126	0.0061
<i>R. hageni</i>	16	9	0.912	37	0.0217	0.0200	0.0088
Duke Forest	13	7	0.973	8	0.0048	0.0048	0.0026
Schenck Forest	3	2	0.667	1	0.0015	0.0015	0.0015

N is the no. colonies studied; only one haplotype was present in each colony.

$\pi$ , nucleotide diversity as determined by the avg no. pairwise nucleotide differences per site;  $\theta = 2N_f\mu$ , where  $N_f$  is the effective population size of females and  $\mu$  is the mutation rate.

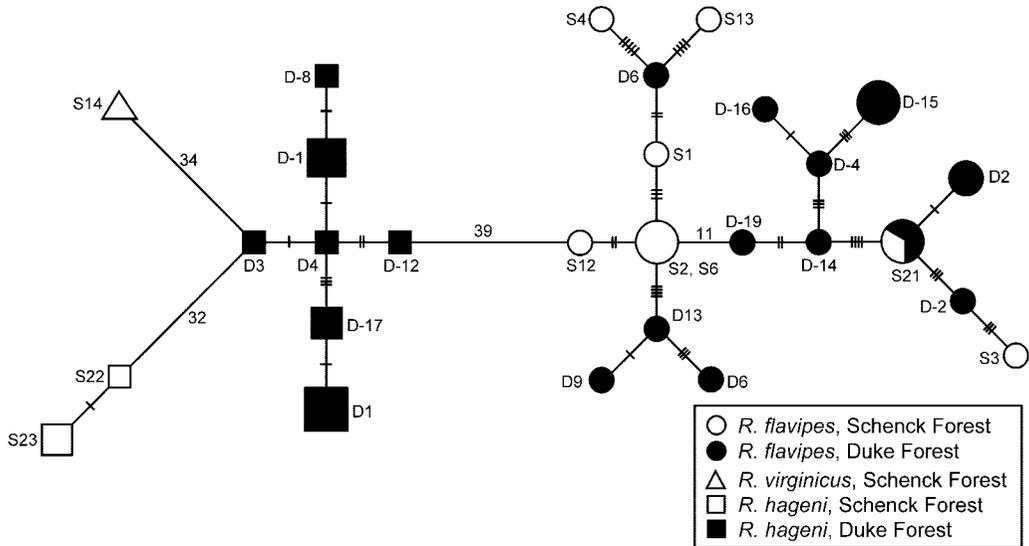


Fig. 3. Minimum spanning trees for *COII* gene sequences for the study colonies. Symbol sizes are proportional to the number of haplotypes depicted. Each dash represents one base pair difference; for many differences, the number of base pair differences is given.

ductives. Simple families made up slightly less than one half of all the *R. flavipes* colonies sampled, and this proportion was similar in the two transects. A little >60% of the *R. hageni* colonies were simple families, including all three of the Schreck Forest colonies. The difference in the frequency of simple and extended family colonies in the two species was not significant ( $P = 0.51$ , Fisher exact test).

Because there was significant differentiation between sites in *R. hageni* ( $F_{ST}$  between transects = 0.19; see Population Genetic Structure), analyses were performed on each population separately to avoid inflation of  $F_{IT}$  due to higher-level structure. Colonies of *R. flavipes* from the two transects were pooled because there was no significant differentiation at this level ( $F_{ST} = 0.01$ ; see Population Genetic Structure). Workers of *R. flavipes* were not significantly inbred relative to the total population ( $F_{IT} = 0.04$ ;  $P = 0.19$ , one-sample  $t$ -test) and were as related as full sibs ( $r = 0.50$ ; Table 7). The  $F$ -statistics and relatedness values for workers in simple family colonies were very similar to those expected for colonies headed by monogamous pairs of unrelated primary reproductives (Table 7, case A), and in no cases did the values differ significantly from the expected (all  $P \geq 0.07$ , one-sample

$t$ -tests). The  $F$ -statistics and relatedness values for the extended family colonies were not significantly different from the values obtained for simple families (all  $P \geq 0.17$ , one-sample  $t$ -tests), and all of the  $F$ -statistics were significantly lower than expected for any of the simulated colony breeding systems involving three or more neotenic reproductives (all  $P \leq 0.01$ , one-sample  $t$ -tests). The lower than expected level of inbreeding in individuals relative to their colony ( $F_{IC}$ ) strongly suggests that the effective number of reproductives in these colonies is small, although there must have been at least three reproductives present in each of these colonies as evidenced by the number of worker genotypes present.

Close inspection of the genotypes present in the two mixed *R. flavipes* colonies revealed the presence of different family groups. Three distinct family groups comprised of 62, 28, and 4 individuals could be discerned in colony S2 (Table 5). All three family groups had genotypes consistent with simple families, but the ratios of the genotypes differed significantly from those expected ( $P < 0.05$ , G-test) only in the larger group (Table 5, Family 1). There was a fourth group of six individuals that did not share any alleles in common with any of the three family groups at locus

Table 4. Family composition of colonies of *R. flavipes* and *R. hageni* in two North Carolina Piedmont sites

Species	Schreck Forest				Duke Forest				Total			
	No. colonies	No. simple family colonies	No. extended family colonies	No. mixed family colonies	No. colonies	No. simple family colonies	No. extended family colonies	No. mixed family colonies	No. colonies	No. simple family colonies	No. extended family colonies	No. mixed family colonies
<i>R. flavipes</i>	9	4 (44.4%)	3 (33.3%)	2 (22.2%)	16	7 (43.8%)	9 (56.3%)	0	25	11 (44.0%)	12 (48.0%)	2 (8.0%)
<i>R. hageni</i>	3	3 (100%)	0	0	13	7 (53.8%)	6 (46.2%)	0	16	10 (62.5%)	6 (37.5%)	0

**Table 5.** Genotypes of different family groups within *R. flavipes* colony S2 at locus *Rf 24-2*

Genotype	Family 1 (n = 62)	Family 2 (n = 28)	Family 3 (n = 4)	Others (n = 6)
134/197		14		
140/212			4	
143/197	16			
143/212	15			
158/182				1
170/197	16			
170/212	15			
176/188				1
182/194				1
191/194				1
194/194				1
194/212				1
197/197		14		

*Rf 24-2*. Thus, this group could not be placed in any of the other families nor did it form an obvious family group by itself. The average degree of relatedness among workers within families was greater than that for full sibs and was significantly greater than relatedness among workers in the entire colony without respect to family ( $P < 0.03$ , *t*-test; Table 8) and significantly greater than relatedness between workers belonging to different families ( $P < 0.005$ , *t*-test). The genotypes of workers in the two families present in colony S6 are shown for locus *Rf 24-2* in Table 6. Family 1 was classified as an extended family because of the presence of more genotypes than is possible with a single set of parents at *Rf 1-3* and *Rf 21-1*, whereas a simple family structure could not be ruled out for family 2. The average relatedness within each of these families was only slightly higher than within the colonies as a whole (Table 8), no doubt because of the large disparity in the number of workers from the two putative families (92 versus 8), and neither value differed significantly from  $r = 0.50$ , the value expected for full sibs (both  $P \geq 0.09$ , one-sample *t*-tests). Although the between family relatedness in this colony was positive ( $r = 0.19$ ), it was not significant ( $P > 0.2$ , one-sample *t*-test). Interestingly, individuals from all groups in both colonies S2 and S6 had the same *COII* haplotype, which was shared in common with one other colony, making it the most common haplotype with a frequency of 0.33.

The *F*-statistics and relatedness values obtained for both *R. hageni* populations are given in Table 7, but

**Table 6.** Genotypes of two different family groups within *R. flavipes* colony S6 at locus *Rf 24-2*

Genotype	Family 1 (n = 92)	Family 2 (n = 8)
128/158	23	
128/188	20	
140/179		3
140/194		3
143/179		1
143/194		1
158/188	22	
188/188	27	

because of the small sample size in the Schenck Forest population, only the results of the Duke Forest population is discussed below. Compared with their *R. flavipes* counterparts, workers from the Duke Forest *R. hageni* colonies were significantly inbred ( $F_{IT} = 0.19$ ;  $P < 0.05$ , approximate *t*-test). In addition, the Duke Forest *R. hageni* population had a significantly higher value of  $F_{CT}$  and relatedness than the *R. flavipes* colonies (both  $P \leq 0.03$ , approximate *t*-test). Workers in simple family colonies from the Duke Forest population of *R. hageni* were significantly more inbred relative to the total population ( $F_{IT} = 0.14$ ;  $P < 0.02$ , one-sample *t*-test) than expected for colonies headed by outbred primary reproductives (Table 7, case A). In addition, the  $F_{CT}$  and relatedness values were significantly higher than expected, suggesting that the reproductives in these colonies were close relatives (both  $P \leq 0.01$ , one-sample *t*-test). The *R. hageni* simple families were more inbred than those of *R. flavipes*;  $F_{IT}$ ,  $F_{CT}$ , and  $r$  values were significantly higher than the corresponding values in *R. flavipes* (all  $P \leq 0.05$ , approximate *t*-test.)

In Duke Forest, workers in *R. hageni* extended family colonies were more inbred ( $F_{IT} = 0.24$ ) and slightly more related ( $r = 0.63$ ) than workers in simple family colonies, but these differences were not significant (both  $P \geq 0.20$ , approximate *t*-test). The number of genotypes present in the extended family colonies indicates that there were more than two reproductives present so that breeding structures with a single pair of neotenic (Table 7, cases B1 and B2) can be ruled out as possibilities for these colonies. The strongly negative value of the coefficient of inbreeding in individuals relative to their colony ( $F_{IC} = -0.26$ ;  $P < 0.01$ , one-sample *t*-test) suggests low numbers of neotenic that were inbred for very few generations (e.g., Table 7, case B3). Although all of the inbreeding coefficients were higher in the *R. hageni* extended family colonies than in those of *R. flavipes*, these differences were not significant (all  $P > 0.10$ , approximate *t*-test).

Analysis of the reconstructed microsatellite genotypes of reproductives gave results that were consistent with those of the worker genotypes. The reproductives in *R. flavipes* were not inbred ( $F_{IT} = -0.01$ ;  $P > 0.5$ , randomization test in FSTAT), and the king and queen within simple families were not significantly related on average ( $r = 0.03$ ,  $P = 0.26$ , one-sample *t*-test). The situation was somewhat different in *R. hageni*. Again, because of the small sample size from Schenck Forest, colonies from this population were not included in the analyses, but they showed the same pattern as those from Duke Forest. Although the reproductives in the Duke Forest simple family colonies were not significantly inbred ( $F_{IT} = 0.03$ ;  $P > 0.3$ , randomization test in FSTAT), cohabiting reproductives were significantly related ( $r = 0.28$ ,  $P < 0.02$ , one-sample *t*-test) and significantly more so than the nestmate reproductives of *R. flavipes* ( $P < 0.04$ , *t*-test). The higher degree of relatedness among reproductives in *R. hageni* may have been caused by a slightly greater frequency of matings between close relatives. Results of analysis with kinship indicated that 2 of 11

**Table 7.** *F*-statistics and relatedness coefficients (mean ± SE) for nestmate workers of *R. flavipes* and *R. hageni* from two study sites in central North Carolina

Species/Site	$F_{IT}$	$F_{CT}$	$F_{IC}$	$r$
<i>R. hageni</i>				
Shenck Forest				
All colonies (all simple families $n = 3$ )	0.223 (±0.197)	0.436 (±0.143)	-0.376 (±0.038)	0.716 (±0.121)
Duke Forest				
All colonies ( $n = 13$ )	0.190 (±0.069)	0.365 (±0.045)	-0.277 (±0.048)	0.614 (±0.046)
Simple family colonies ( $n = 7$ )	0.140 (±0.053)	0.332 (±0.024)	-0.289 (±0.035)	0.595 (±0.024)
Extended family colonies ( $n = 6$ )	0.236 (±0.097)	0.394 (±0.079)	-0.257 (±0.079)	0.599 (±0.092)
<i>R. flavipes</i>				
All colonies ( $n = 25$ )	0.037 (±0.040)	0.244 (±0.024)	-0.274 (±0.026)	0.498 (±0.029)
Simple family colonies ( $n = 11$ )	-0.002 (±0.045)	0.249 (±0.023)	-0.336 (±0.023)	0.531 (±0.027)
Extended family colonies ( $n = 12$ )	0.056 (±0.092)	0.275 (±0.027)	-0.302 (±0.040)	0.487 (±0.036)
Mixed family colonies ( $n = 2$ )	0.060 (±0.069)	0.198 (±0.038)	-0.174 (±0.049)	0.396 (±0.055)
Simulated breeding system				
Colonies headed by outbred reproductive pairs	0.00	0.25	-0.33	0.50
Colonies headed by inbreeding neotenic				
(1) $N_f = N_m = 1, X = 1$	0.33	0.42	-0.14	0.62
(2) $N_f = N_m = 1, X = 3$	0.57	0.65	-0.22	0.82
(3) $N_f = 2, N_m = 1, X = 3$	0.52	0.59	-0.17	0.75
(4) $N_f = N_m = 10, X = 1$	0.33	0.34	-0.01	0.51
(5) $N_f = N_m = 10, X = 3$	0.37	0.38	-0.02	0.56
(6) $N_f = 200, N_m = 100, X = 3$	0.33	0.34	-0.00	0.50

*R. flavipes* colonies from the two sites were pooled because there was no significant differentiation between the sites. Also included are values expected for several possible breeding systems as derived from computer simulations by Thorne et al. (1999) and Bulmer et al. (2001). For the simulated breeding systems, X represents the no. of generations of production of neotenic reproductives within a colony;  $N_f$  and  $N_m$  represent the no. replacement females and males, respectively, produced per generation.

pairs of *R. flavipes* reproductives (18%) were putative full sibs ( $P < 0.05$ ), whereas four of the seven *R. hageni* simple families (57%) were likely headed by full siblings ( $P < 0.05$ ), although the difference in this frequency was not significant ( $P = 0.07$ , Fisher exact test). Alternatively, such colonies could have been headed by one primary reproductive and one replacement neotenic.

**Population Genetic Structure**

The  $F_{ST}$  and  $Nm$  values between the Shenck and Duke populations for both the microsatellite data and the mtDNA data are shown in Table 9. In *R. flavipes*, there was no significant differentiation between the two locations according to the microsatellite genotypes of either the workers ( $F_{ST} = -0.00$ ; 95% CI = -0.01-0.00) or the inferred genotypes of reproductives ( $F_{ST} = -0.01$ ; 95% CI = -0.03-0.01). In contrast, there was significant differentiation between locations in *R. hageni* using both worker microsatellite genotypes ( $F_{ST} = 0.19$ ; 95% CI = 0.10-0.28) and the reconstructed genotypes of reproductives ( $F_{ST} = 0.24$ ; 95% CI = 0.14-0.37), but because of the small sample

size in the Shenck Forest population ( $n = 3$ ), this conclusion should be taken as tentative. Results of the analysis of the mtDNA data showed greater degrees of differentiation. This value was considerably higher in *R. hageni* than in *R. flavipes*, with effective number of migrants ( $Nm$ ) in the former species below 0.1, whereas  $Nm$  was above one in *R. flavipes*.

The data from the *COII* gene conformed to neutrality, according to the test of selective neutrality of Tajima (1989), for both *R. flavipes* and *R. hageni* (both  $P > 0.1$ ). The *R. flavipes* sequences formed a single well-supported clade in the neighbor-joining tree, within which were located *R. flavipes* haplotypes from GenBank (Fig. 2). Although the haplotypes from the two populations were not completely separated into different clades, some segregation between them can be seen in the neighbor-joining tree and the minimum spanning tree (Fig. 3). The *R. hageni* haplotypes formed two well-supported clades with the two populations clearly separated from each other. There was only a single *COII* sequence for *R. hageni* available from GenBank, and this haplotype showed strong affinity to the haplotypes from the Shenck Forest population (Fig. 2). The *R. virginicus* haplotype and the *R.*

**Table 8.** Relatedness ( $r$ ) of workers in two mixed family colonies of *R. flavipes*

Colony	No. families present	$r$ among all workers	$r$ between workers in the same family	$r$ between workers in different families
S2	3 <sup>a</sup>	0.346 (SE = 0.0842) (95% CI = 0.2059)	0.600 (SE = 0.068) (95% CI = 0.1663)	0.154 (SE = 0.119) (95% CI = 0.291)
S6	2	0.439 (SE = 0.056) (95% CI = 0.2109)	0.481 (SE = 0.078) (95% CI = 0.1919)	0.185 (SE = 0.133) (95% CI = 0.325)

<sup>a</sup> There was also a group of 6 individuals that did not form an obvious family group.

*virginicus* GenBank sequence formed a well-supported clade within the tree with strong affinity to the Duke Forest haplotypes. The minimum spanning trees showed similar relationships, with the *R. virginicus* haplotype embedded within the *R. hageni* clade (Fig. 3).

We can gain some insights into the relative effective population sizes from analysis of mtDNA diversity.  $\theta$ , as shown in Table 3, is a function of the effective population size of females and the mutation rate. Assuming that the *COII* gene has the same mutation rate and has reached mutation-drift equilibrium in the different populations and species, variation in the magnitude of  $\theta$  should reflect differences in effective population size. Of the three populations with sufficient numbers to analyze, the two *R. flavipes* populations were the largest (mean  $\theta = 0.013 \pm 0.006$  for both Schenck Forest and Duke Forest). The Duke Forest population of *R. hageni* was considerably smaller ( $\theta = 0.005 \pm 0.003$ ), amounting to only about 35% of the *R. flavipes* population from the same site ( $P < 0.0005$ , *t*-test).

### Isolation by Distance

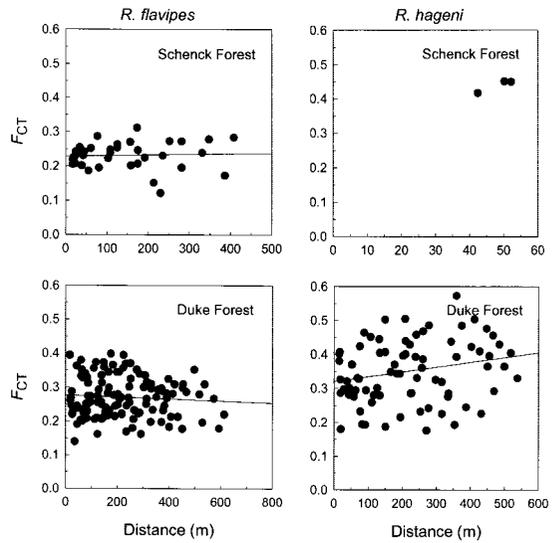
There was no significant correlation between geographic distance and  $F_{CT}$  for *R. flavipes* (Fig. 4;  $r = 0.06$  and  $-0.07$  for Schenck and Duke transects, respectively; both  $P \geq 0.27$ ). However, in *R. hageni* there was a significant correlation in the Duke Forest transect (Fig. 4;  $r = 0.22$ ,  $P < 0.03$ ), but not in the Schenck Forest transect (Fig. 4;  $r = 0.97$ ,  $P = 0.48$ ) where there were only three colonies.

If colony reproduction by budding were common, colonies located near each other should have the same mtDNA haplotype, because female reproductives in daughter colonies are expected to develop from within the colony. However, only two *R. flavipes* colonies in Schenck Forest shared the same haplotype;

**Table 9.** Genetic differentiation ( $F_{ST}$ ) and effective migration rates (Nm) between two populations each of *R. flavipes* and *R. hageni* using nuclear (microsatellite) and mtDNA markers

	$F_{ST}$	Nm
<i>R. flavipes</i>		
Microsatellites		
Worker genotypes	-0.000	NA
(95% CIs)	(-0.010 to 0.000)	
Genotypes of reproductives	-0.010	NA
(95% CIs)	(-0.030 to 0.010)	
mtDNA COII	0.217	1.804
( <i>P</i> )	( <i>P</i> < 0.010)	
<i>R. hageni</i>		
Microsatellites		
Worker genotypes	0.190	1.066
(95% CIs)	(0.100-0.280)	
Genotypes of reproductives	0.240	0.792
(95% CIs)	(0.140-0.370)	
mtDNA COII	0.923	0.042
( <i>P</i> )	( <i>P</i> < 0.002)	

For the microsatellite data, Nm is calculated from  $F_{ST}$  by the equation  $F_{ST} = 1/(4Nm + 1)$  after Wright (1931), whereas for the mtDNA markers, Nm is calculated from  $F_{ST} = 1/(2Nm + 1)$  because of the lower effective population size associated with maternally inherited haploid genes (McCauley 1998).



**Fig. 4.** Relationship between genetic differentiation ( $F_{CT}$ ) based on microsatellite genotypes and physical distance among pairs of conspecific colonies within each transect. Regression lines are described by  $y = 1.85x + 0.229$  for Schenck Forest *R. flavipes*,  $y = -2.89x + 0.277$  for Duke Forest *R. flavipes*, and  $y = 1.40x + 0.320$  for Duke Forest *R. hageni*.

these were located 55 m apart and had 31 private alleles between them. In the Duke Forest *R. flavipes* populations, there were two pairs of colonies and a set of three colonies who shared the same haplotypes. However, in only one case were colonies with the same haplotype located adjacent to each other, and even in this case the average relatedness between workers in the two colonies was  $r = -0.10$ , indicating that these two colonies were no more related to each other than they were to other colonies from the same population. Overall, the average relatedness between *R. flavipes* workers in colonies sharing the same haplotype was no greater than it was between workers with different haplotypes ( $r = 0.071 \pm 0.054$  and  $-0.020 \pm 0.117$ , for same and different haplotypes, respectively;  $t_{118} = 0.97$ ,  $P > 0.3$ ). Similarly, two of the *R. hageni* colonies in Schenck Forest had the same haplotype, but the workers in these colonies were unrelated to each other ( $r = -0.147$ ). In the Duke Forest *R. hageni* population, there were two haplotypes shared by more than one colony (two colonies in one case and three in the other), but same haplotype colonies were no more related to each other than they were to colonies with different haplotypes ( $r = -0.009 \pm 0.087$  and  $-0.001 \pm 0.151$ , for same and different haplotypes, respectively;  $t_{79} = -1.25$ ,  $P > 0.9$ ).

### Discussion

Results of this study, the first detailed comparative study of colony and population genetic structure of two sympatric subterranean termite species, reveal

important differences and similarities between the breeding systems of *R. flavipes* and *R. hageni* in central North Carolina. Both species had high proportions of simple family colonies: some 44% in *R. flavipes* and 63% in *R. hageni*. Colonies of *R. flavipes* were generally founded by pairs of outbred primary reproductives, whereas those of *R. hageni* appeared to be frequently founded by close relatives resulting in colonies that were significantly inbred. About one-half the *R. flavipes* colonies in the study population, and almost 40% of the *R. hageni* colonies, were extended families likely headed by low numbers of neotenic reproductives, either by themselves or together with the one or both primaries. A small percentage of *R. flavipes* colonies (8%) was composed of a mixture of two or more different families, although the majority of individuals in these colonies were from a single family. In contrast, we did not find any mixed family colonies of *R. hageni* at either study site.

The proportion of simple family colonies of *R. flavipes* found in this study is lower than that reported in previous studies of this species from these same and other nearby locations. In a similar transect study of 56 colonies from Schenck Forest, Duke Forest, and another central North Carolina wooded area, Vargo (2003a) found that about three-quarters of the colonies were simple families and no mixed family colonies were observed. DeHeer and Vargo (2004) studied 30 *R. flavipes* colonies from Schenck Forest and a nearby forest site and found that 70% were simple families, 27% were extended families, and one colony was comprised of a mixture of different families. The reason for the lower proportion of simple family colonies found in this study is not obvious, but may be caused by the sampling method used. In the previous two studies, all termites encountered were collected, whereas in this study we attempted to focus on the slightly smaller species, *R. virginicus*, so worker groups that had conspicuously large body sizes were not collected. If simple family colonies of *R. flavipes* tend to have larger workers than extended family colonies at these study sites, the samples of this species studied here may have been biased toward extended families. Nevertheless, the genetic structure of simple family colonies was consistent among all three studies, as well as another study conducted in an urban habitat in central North Carolina (Vargo 2003b) and a study in Massachusetts (Bulmer et al. 2001), providing strong support to the conclusion that established simple families are generally headed by outbred primary reproductives. However, as discussed below, recent evidence suggests that sib pairings among colony founders may be fairly common in *R. flavipes*, but that they are selected against and relatively few survive in established colonies (DeHeer and Vargo 2005).

We found two mixed family colonies of *R. flavipes*, both of which were located in Schenck Forest. Such colonies of this species have been found in other studies (Jenkins et al. 1999, Bulmer et al. 2001, DeHeer and Vargo 2004), where they also comprised a small fraction of the total colonies present. In a detailed study of colony foraging areas over time, DeHeer and

Vargo (2004) documented the fusion of two distinct colonies in Schenck Forest, the same location where the two mixed-family colonies were found in this study. We collected our samples in this study during a single point in time and are therefore unable to say whether the mixed family colonies studied here arose through colony fusion. Nonetheless, the fact that nearly all of the 100 individuals genotyped in each colony could be assigned to a distinct family with no indication of interbreeding between the reproductives heading each family is consistent with a fusion event involving two or more colonies.

Surprisingly, we found that all the families present within each of the mixed family colonies shared the same mtDNA haplotype. This may suggest that these mixed family colonies are comprised of distantly related kin, a conclusion suggested by the moderate degree of relatedness among families within the same colony ( $r = 0.15$  and  $0.18$ ), although these values were not significantly different from zero. The probability that the different families present in these colonies would have the same mtDNA haplotype by chance are less than 1 in 10 in the case of colony S6 and less than 3 in 1,000 in the case of colony S2. However, mixed family colonies of *R. flavipes* are not always comprised of individuals with the same mtDNA haplotypes; in fact Bulmer et al. (2001) and Jenkins et al. (1999) used variation in mtDNA to detect the presence of multiple matrilineal lines within *R. flavipes* colonies. The combined results of all these studies indicate that a small proportion of colonies of *R. flavipes* in several populations consist of a mixture of different families that may or may not share the same mtDNA haplotype and that the combined use of sensitive markers of both the mitochondrial and nuclear genomes can be a powerful means to uncover the complexities of colony breeding structure in subterranean termites.

The apparent lack of interbreeding among reproductives from different families in these colonies suggests that the presence of multiple families within colonies may be a temporary phenomenon, with reproduction eventually being monopolized by a single family. Indeed, ongoing studies of *R. flavipes* colonies that were observed to fuse in the field (DeHeer and Vargo 2004) indicate that the newly produced larvae present 1 yr after fusion are all the progeny of the reproductives from only one of the original colonies, and the proportion of workers in this original colony has increased significantly (C. DeHeer and E. Vargo, unpublished data). Similarly, a laboratory study in which two colonies of *R. flavipes* were observed to fuse indicated that reproduction within fused colonies is limited to individuals of only one of the original colonies (Fisher et al. 2004).

There is very little previous information on the breeding system of *R. hageni*, and our results on colony genetic structure of this species are the first of which we are aware. Our results show that most colonies in the study population were each headed by a monogamous pair of reproductives. The male and female reproductives in colonies tend to be closely related ( $r = 0.28$ ). There are two possible scenarios leading to a

high frequency of consanguineous matings: (1) related primary reproductives tend to pair during mating flights or (2) a sizable portion of colonies are headed by neotenic, either a single pair or one neotenic together with one of the original primaries. Our data provide indirect evidence for the first scenario. We found significant isolation by distance in the Duke Forest population of *R. hageni* using the microsatellite markers, and a strong suggestion of it in the Schenck Forest population of this species in which only three colonies were sampled. Such population viscosity is consistent with short range mating flights of primary reproductives and/or frequent colony reproduction by budding. This latter possibility can be excluded based on the distribution of the mtDNA haplotypes. Frequent budding should produce groups of nearby colonies with the same haplotype, but there was no evidence of such a pattern. Thus, the most likely explanation for population viscosity is short-range dispersal of primary reproductives, a phenomenon which could also result in close relatives pairing during mating flights. In the few mating flights that we have observed in this species (unpublished data), alates flew at a height of no more than 3 m and quickly descended near their point of emergence. Two other findings were consistent with short-range dispersal of reproductives in *R. hageni*. First, the apparently lower effective population size in the Duke population compared with sympatric *R. flavipes* suggests populations of *R. hageni* have greater genetic structure locally, as would be expected if dispersal were more limited. And second, the strong genetic differentiation between the Schenck Forest and Duke Forest populations, leading to an effective number of migrants ( $N_m$ ) well below the value of 1.0 thought to be the threshold for preventing evolutionary divergence by drift (Wright 1931), suggests very limited gene flow at spatial scales of 30 km and possibly less. In fact, the actual value of  $N_m$  may be lower than this estimate. This is because  $N_m$  is inversely related to  $F_{ST}$ , the magnitude of which is dependent on the level of genetic variation, being lower in highly variable markers such as microsatellites (Hedrick 2005). An alternative explanation for the high  $F_{ST}$  between the two *R. hageni* populations, however, is that they contain different species or subspecies as discussed below.

Our results suggest that primary reproductives heading established simple family colonies in *R. hageni* are on average more closely related than those of *R. flavipes* and that this is most likely caused by a higher frequency of sibling reproductive pairs in the former species (60% versus 20%). The actual proportion of probable consanguineous matings in the study population of *R. flavipes* is probably lower; in a more extensive study of established simple family colonies from these and nearby populations, DeHeer and Vargo (2005) found that only 11 of 243 (5%) were headed by probable sibling primary reproductives. However, these authors also reported a significantly higher proportion of sibling pairs present among newly formed tandem pairs of *R. flavipes* during mating flights (18 of 65; 28%), suggesting such pairs in this

species experience low survivorship because of inbreeding depression. No comparable study of *R. hageni* tandem pairs exists, but our data suggest that nearly 60% of the simple family colonies are headed by sibling pairs. Thus, in the study population, either the cost of inbreeding in *R. hageni* colonies is less than *R. flavipes* or the former species has fewer opportunities for outbreeding.

Of the 25 *R. flavipes* colonies sampled in this study, only a single colony spanned more than a single collection site. The occurrence of spatially localized colonies of this species is consistent with the results of other studies from central North Carolina (Vargo 2003a, b, DeHeer and Vargo 2004) and coastal South Carolina (E. Vargo, C. DeHeer, and T. Juba, unpublished data), where colonies generally had foraging areas <30 linear m in diameter. However, in this study, we found one colony that spanned 83 linear m, surpassing the previous records of 79 and 71 linear m for this species (Grace et al. 1989, Su 1994). Interestingly, the one *R. virginicus* colony sampled spanned at least 120 linear m, which is a record for a colony of any *Reticulitermes* spp., rivaling the foraging distances of the Formosan subterranean termite, *C. formosanus*, several colonies of which have been documented to forage over 100 m (Su and Scheffrahn 1988, Su 1994, Messenger and Su 2005, Vargo et al. 2005) up to a maximum of 185 m (Su 1994). Similarly expansive colonies of *R. virginicus* have been reported previously from central North Carolina (Vargo 2003b, DeHeer and Vargo 2004), suggesting that this may be a common trait of this species. All of the *R. hageni* colonies in this study were sampled at only a single point, indicating they are relatively localized. This conclusion is supported by the results of recent studies of this species in the Coastal Plain of North Carolina and South Carolina (E. Vargo, C. DeHeer, T. Juba, unpublished data), in which all 49 colonies sampled at 15-m intervals were present at only a single location. In summary, available evidence shows both intra- and interspecific variation in the spatial expanse of *Reticulitermes* spp. colonies, but the causes of this variation remain to be identified.

*Reticulitermes flavipes*, *R. hageni*, and *R. virginicus* occur sympatrically over much of their ranges in the southeastern United States (Snyder 1954). This, together with the fact that all three species appear to play similar ecological roles, suggests some degree of resource partitioning among them. The relative distribution and activity of these species in a given location is most likely influenced by variation in soil moisture and temperature, the two major factors determining the local distribution of termite species (Kofoid 1934). Evidence in support of such a role for temperature and moisture in resource partitioning among sympatric *Reticulitermes* spp. comes from a recent study of seasonal foraging activity of *R. flavipes* and *R. hageni* in central Texas, in which *R. flavipes* was found to be most active during the cooler, wet months and *R. hageni* during the warmer, dry months (Houseman et al. 2001). In southern Mississippi, Howard et al. (1982) found colonies of *R. virginicus* more commonly

in lower, wetter areas than colonies of *R. flavipes*. No effort was made in this study to relate species distribution to seasonal activity or to variation in soil moisture, but this is an area that certainly deserves attention in future studies.

There have been very few comparative studies of colony and population genetic structure of closely related termite species. Thompson and Hebert (1998a) studied the breeding system of *Nasutitermes nigriceps* and *N. costalis* in Jamaica using allozymes and restriction fragment-length polymorphism analysis of the A+T-rich region of mtDNA. Nearly all (95%) colonies of *N. nigriceps* were simple families, whereas the few remaining colonies appeared to be extended families descended from simple families. However, these authors could not draw conclusions concerning the breeding system of *N. costalis* in the study populations because there was no variation in either the allozyme or mtDNA markers, possibly because of recent colonization of Jamaica by this species. Thompson and Hebert (1998a) assumed that most colonies of *N. costalis* species were headed by multiple reproductives because a study based on nest dissections of populations on other Caribbean islands (Roisin and Pasteels 1986) found that all 14 colonies examined contained multiple queens. Clément and coworkers (Clément 1981, Clément et al. 2001) report variation in the breeding systems among and within several *Reticulitermes* spp. in Europe based on variation at two allozyme loci, but the small number of alleles present in most populations in these studies (mean = 2.3 alleles/locus, range = 1–5) did not provide sufficient sensitivity for detailed analysis of colony genetic structure.

The lack of genetic differentiation between the two *R. flavipes* populations is consistent with results from previous findings, in which no or very weak differentiation was detected at scales of 0.5–100 km across a broad part of the geographic range of this species (Reilly 1986, 1987; Bulmer et al. 2001; Vargo 2003a). The strong genetic differentiation ( $F_{ST}$  for microsatellites  $\approx 0.2$ ;  $\theta_{ST}$  for mtDNA  $\approx 0.9$ ) between the two *R. hageni* populations separated by only 27 km would appear to be unusual for termites. In a study of *N. nigriceps*, Thompson and Hebert (1998b) found moderate levels of differentiation ( $F_{ST} = 0.1$ ) among Jamaican populations separated by distances of 100–200 km. Goodisman and Crozier (2002) estimated  $F_{ST}$  values of 0.51 and 0.37 for the primitive termite *Mastotermes darwiniensis* for spatial scales of 2–350 and  $\approx 1,500$  km, respectively, using microsatellites.

The presence of strong population structure in *R. hageni* should provide greater opportunity for local evolution in this species. Accordingly, *R. hageni* shows much greater variability in cuticular hydrocarbon composition than other North American *Reticulitermes* species, including *R. flavipes*. In a study of *Reticulitermes* spp. samples from a large portion of the United States, Page et al. (2002) identified 26 distinct cuticular hydrocarbon phenotypes, 11 (42%) of which were identified as *R. hageni* based on soldier morphology. In contrast, there were only five phenotypes cor-

responding to *R. flavipes*. Page et al. (2002) suggested that these different phenotypes represent different species or subspecies, although a phylogenetic study by Jenkins et al. (2000) did not support the separation of different cuticular hydrocarbon phenotypes of *R. flavipes* into distinct taxa. Cuticular hydrocarbon analysis was not performed on the samples used in this study so we are unable to say whether the strong genetic differentiation between the Duke and Schenck Forest populations of *R. hageni* correspond to differences in cuticular hydrocarbon phenotypes. However, a recent study using both mtDNA 16S rDNA sequence data and morphological characters suggests that *R. hageni* in the far eastern United States consists of two species (J. Austin, A. Szalanski, R. Scheffrahn, and M. Messenger, unpublished data), and an analysis of the 16S gene in samples from this study suggest that the Schenck Forest population is *R. hageni sensu stricto*, whereas the Duke Forest population is *R. n. sp.* (J. Austin and E. Vargo, unpublished data). However, because *R. hageni* has not yet been formally split, we have treated it as a single species in this study. Because the two populations were analyzed separately in our study, eventual splitting of the populations into different species will not significantly affect the conclusions presented here regarding colony breeding structure and dispersal.

In conclusion, these results reveal some important similarities and differences in the breeding systems of *R. flavipes* and *R. hageni*, two ecologically similar species that are sympatric over much of the southeastern United States. Colonies of both species appear to be founded by pairs of primaries. A substantial portion of the colonies in both species is comprised of simple families, presumably still headed by the original founding pair, with the remainder of the colonies consisting mainly of extended families headed by low numbers of neotemics, and a small proportion of *R. flavipes* colonies comprised of mixed families most likely arising from colony fusion. One of the main differences between these species appears to be the distance over which they disperse. The evidence suggests that *R. hageni* disperses over relatively short distances, resulting in (1) greater levels of inbreeding in simple family colonies caused by the pairing of relatives, (2) smaller effective population size, and (3) significant structuring of local populations. Such limited dispersal may allow for greater degrees of adaptation to local ecological conditions and a higher rate of speciation.

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