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HIERARCHICAL ANALYSIS OF COLONY AND POPULATION GENETIC STRUCTURE OF THE EASTERN SUBTERRANEAN TERMITE, *RETICULITERMES FLAVIPES*, USING TWO CLASSES OF MOLECULAR MARKERS

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Abstract.—Termites (Isoptera) comprise a large and important group of eusocial insects, yet, in contrast to the eusocial Hymenoptera (ants, bees, wasps), the breeding systems of termites remain poorly understood. In this study, I inferred the breeding system of the subterranean termite *Reticulitermes flavipes* based on colony and population genetic structure as determined from microsatellite and mitochondrial DNA markers. Termites were sampled from natural wood debris from three undisturbed, forested sites in central North Carolina. In each site, two transects separated by 1 km were sampled at approximately 15-m intervals. A total of 1272 workers collected from 57 collection points were genotyped at six microsatellite loci, and mitochondrial DNA haplotype was determined for a subset of these individuals using either restriction fragment length polymorphism or sequence variation in the AT-rich region. Colonies appeared to be localized: workers from the 57 collection points represented 56 genetically distinct colonies with only a single colony occupying two collection points located 15 m apart. Genetic analysis of family structure and comparisons of estimates of *F*-statistics (F_{IT}, F_{IC}, F_{CT}) and coefficients of relatedness (r) among nestmate workers with results of computer simulations of potential breeding systems suggested that 77% of all colonies were simple families headed by outbred monogamous pairs, whereas the remaining colonies were extended (inbred) families headed by low numbers of neotenics (about two females and one male) who were the direct offspring of the colony founders. There was no detectable isolation by distance among colonies along transects, suggesting that colony reproduction by budding is not common and that dispersal of reproductives during mating flights is not limited over this distance. Higher-level analysis of the microsatellite loci indicated weak but significant differentiation among sites ($F_{ST} = 0.06$), a distance of 16–38 km, and between transects within sites ($F_{ST} = 0.06$), a distance of 1 km. No significant differentiation at either the transect or site level was detected in the mitochondrial DNA sequence data. These results indicate that the study populations of *R. flavipes* have a breeding system characterized by monogamous pairs of outbred reproductives and relatively low levels of inbreeding because most colonies do not live long enough to produce neotenics, and those colonies that do generate neotenics contain an effectively small number of them.

Key words.—Breeding structure, gene flow, microsatellites, mitochondrial DNA sequence data, social organization.

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The populations of most species exhibit some level of genetic structure, and these patterns of population subdivision are central to many key issues in evolutionary biology (Barton and Clark 1990). Population genetic structure arises through departures from panmixia, most often due to the tendency of individuals to mate with others within the same subpopulation. However, even within subpopulations breeding is often nonrandom, resulting in hierarchically structured populations. Thus, knowledge of an organism's breeding system and dispersal behavior is critical for understanding observed patterns of population genetic structure and the processes responsible for producing these patterns (Sugg et al. 1996). Increasingly, researchers are using data from molecular markers not only to characterize population subdivision, but to infer attributes of a species' breeding system and dispersal behavior that generate such structure (Avise 1994; Ross 2001). Of particular interest are studies that combine different classes of markers with alternate modes of inheritance, such as nuclear and mitochondrial DNA (mtDNA) markers, which can help discern the maternal and paternal contributions to gene flow and population structure (e.g., Shoemaker and Ross 1996; Ross and Shoemaker 1997; Ross et al. 1997, 1999; Goodisman and Ross 1998; Latta et al. 1998; Doums et al. 2002; Arnaud-Haond et al. 2003; Ruppell et al. 2003).

Many organisms form social groups with distinct patterns of mating and dispersal that can profoundly affect the partitioning of genetic variation within and among groups (Sugg et al. 1996). With their advanced form of social organization in which breeding is confined to a single or a few individuals in groups whose members may number in the thousands or millions, eusocial insects are excellent models for studies of how social behavior can affect population genetic structure. Compared to the wealth of population genetic studies on the eusocial Hymenoptera (Pamilo et al. 1997; Ross 2001), termites (Isoptera) have received relatively little attention. This despite the fact that termites provide a useful comparative context for investigating the relationship between breeding system and genetic structure in two important ways. First, termites are phylogenetically far removed from the Hymenoptera, providing an independent taxon for understanding how changes in breeding system and dispersal behavior may affect genetic structure in social insects. Second, there is considerable variation in breeding system within and among termite species (Clément 1981; Reilly 1987; Luykx 1993; Atkinson and Adams 1997; Husseneder et al. 1998, 1999, 2002; Thompson and Hebert 1998a,b; Jenkins et al. 1999a; Bulmer et al. 2001; Clément et al. 2001; Husseneder and Grace 2001; Vargo et al. 2003), allowing for comparative studies of the causes and consequences of changes in breeding system properties.

Subterranean termites of the genus *Reticulitermes* are widespread throughout the temperate and subtropical regions of the world (Pearce 1997), where they form cryptic colonies

comprised of diffuse nests and multiple feeding sites connected by underground tunnels. Breeding systems of *Reticulitermes* species can be complex. The predominant mode of colony founding is believed to be by monogamous pairs of primary reproductives following a mating flight (Thorne et al. 1999). Over time, one or both primary reproductives die, resulting in the development of numerous neotenics (nonalate-derived reproductives) that inbreed within the colony. In addition, observations and experimental data suggest that budding, in which groups of workers split from the main colony and establish their own independent daughter colony, may be a common mode of reproduction in *Reticulitermes* species (Esenther 1969; Howard and Haverty 1980; Clément 1981; Myles and Nutting 1988; Grace 1996; Thorne 1998), leading to high population viscosity. Finally, it is possible that unrelated colonies can fuse, or that unrelated reproductives are recruited into established nests (Clément 1981; Jenkins et al. 1999b; Bulmer et al. 2001; Matsuura and Nishida 2001), either of which, if common, would lower relatedness within colonies and decrease the genetic contrast among colonies (Thorne et al. 1999; Bulmer et al. 2001). Thus, the breeding structure within *Reticulitermes* colonies may be influenced by a variety of factors, including age of colony, mode of colony founding, and the dynamics of interactions with neighboring colonies.

There are two published studies of colony and population genetic structure in the eastern subterranean termite, *R. flavipes*, and these give contrasting views of this species' breeding system. Allozyme data from Reilly (1987) suggest that in a central Tennessee study population there was a high level of inbreeding ($F_{IT} = 0.62$) and extensive mixing of workers from different colonies or frequent nest budding with interconnected nests (Thorne et al. 1999; Bulmer et al. 2001). In contrast, Bulmer et al. (2001), using allozyme data and a mtDNA marker, reported lower but still substantial levels of inbreeding ($F_{IT} \approx 0.30$) in a Massachusetts population consisting of a mixture of simple families (27%), extended families derived from simple families (64%), and genetically complex groups containing multiple unrelated female reproductives (9%). The reasons for the differences between the two studies are not clear but could be due to geographic variation in the breeding system of this species and/or differences in the properties of the markers used.

The application of highly variable codominant markers, such as microsatellites, should provide a more detailed picture of the breeding system of *R. flavipes* and other subterranean termites because such markers have greater power for establishing pedigree relationships within colonies, assigning groups of workers to specific colonies, and detecting colonies that may contain multiple unrelated same-sex reproductives. The objective of the present study was to infer the major properties of the breeding system of a population of *R. flavipes* and to characterize the genetic structure at different spatial scales using two classes of highly variable molecular genetic markers. Microsatellite markers (Vargo 2000) were used to determine the kin composition of colonies and to examine population genetic structure at various spatial scales using *F*-statistics. Sequence variation in the adenine-thymine-rich (AT-rich) region of mtDNA was used to investigate the matrifilial associations within colonies and to assess population structure at higher levels. Finally, both the microsatellite and mtDNA markers were used to discern patterns of isolation by distance on a local scale as a means of investigating possible limited dispersal by either or both sexes.

Materials and Methods

Sample Collection

Specimens of *R. flavipes* were collected from three wooded sites in central North Carolina: Lake Wheeler Field Laboratory, a North Carolina State University facility in southern Wake County; Schenck Forest, also a North Carolina State University facility in western Wake County; and Duke Forest, part of Duke University, in Durham County. The Lake Wheeler site consisted of about a 30-year-old mostly loblolly pine (*Pinus taeda*) stand, whereas the other two sites were more than 50-year-old stands of mixed upland hardwoods and loblolly pine. The distances between the Lake Wheeler site and the Schenck Forest and Duke Forest sites were 15.9 km and 37.6 km, respectively. The distance between the Schenck Forest and Duke Forest sites was 27 km. Samples of workers, soldiers, and nymphs or reproductives (when the latter were present) were collected from naturally occurring wood debris in transect fashion in summer 1999. Once a sample was collected, I continued in a set direction for 15 m and began searching for termites. The search was conducted in concentric semicircles away from the previous collection point until termites were found, to maintain a minimum distance of 15 m between collection points. I measured the distance from the previous collection point to the spot along the transect where the subsequent collection was made. If the collection point was located off the transect line, then the perpendicular deviation from the collection point to the transect was also measured. Ten collection points were sampled in each transect. In each site, I sampled two parallel transects located 1 km apart.

Samples were transported alive to the laboratory, where they were frozen and stored at −70°C. To confirm species identity, soldiers were examined using the key of Scheffrahn and Su (1994). Voucher specimens have been deposited in the North Carolina State University (NCSU) Insect Collection.

Genotyping

Whole bodies were pulverized in liquid nitrogen, and total genomic DNA was extracted using either the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) or the DNeasy Tissue Kit (Qiagen, Valencia, CA). Microsatellite genotypes for approximately 20 workers from each collection point were determined by means of polymerase chain reaction (PCR) amplification using one of nine pairs of primers and a fluorescent labeled primer, followed by separation of the PCR products in polyacrylamide sequencing gels run on a Li-Cor 4000 or 4200 automated DNA sequencer (Li-Cor Biosciences, Lincoln, NE). Details for primer development and PCR conditions are given in Vargo (2000). I multiplexed primer pairs 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*; *Rf 3-1* and *Rf 21-1*. I collected a primary reproductive pair from

one collection point at the Schenck Forest site, and these individuals along with 100 nestmate workers were genotyped to confirm Mendelian inheritance of alleles at all of the microsatellite loci. The observed genotype frequencies of the workers were compared with frequencies expected from the parental genotypes by means of a χ^2 test with the Bonferroni correction applied.

PCR–restriction fragment length polymorphism (RFLP) was performed on the AT-rich noncoding region. PCR amplification was performed using the conserved primers SR-J-14612 and TM-N-193, which flank the region (Simon et al. 1994; Jenkins et al. 1999a). Reaction volumes were 10 μl, containing 1.2 μl (about 6 ng) genomic DNA, 10× reaction buffer, 0.2 μg/μl BSA, 2 mM $MgCl_2$, 200 μM each dNTP, 0.4 units Biolase DNA polymerase (Bioline, Canton, MA), and 1 pmol each primer. PCR was performed on a PTC-100 thermal cycler (MJ Research, Inc., Littletown, MA) using the following program: initial denaturation step at 94°C (50 sec) followed by 35 cycles at 94°C (50 sec), 55°C (2 min), and 72°C (2 min), with a final extension step at 72°C (5 min).

PCR products varied in size from about 1500 to 2500 bp. These were double digested according to the manufacturers' instructions, first with *Apo*I (New England Biolabs, Beverly, MA) and then with *Hae*III (Promega). The digested products were run out on 1.5% agarose gels and visualized by ethidium bromide staining. Twenty workers from each collection point were genotyped, as well as the primary king and queen collected from Schenck Forest.

A segment of the AT-rich region was sequenced using the primers TM-N-193 and AT-J-T1, which yields an approximately 380-bp fragment beginning in tRNA methionine and includes tRNA glutamine and tRNA isoleucine extending some 190 bp into the AT-rich region (Jenkins et al. 1999a). PCR amplification using these primers usually produced two products, one at about 380 bp and the other at approximately 1800 bp. When the larger product was gel-extracted and reamplified, it again produced two bands of 380 bp and 1800 bp, indicating a likely duplication of one of the primer sites. Sequencing of the two products with both primers revealed different sequences for AT-J-T1 but identical sequence for TM-N-193, indicating the presence of two sites for the AT-J-T1 primer designed by Jenkins et al. (1999a). Consequently, following PCR and gel separation, the smaller band was excised and then extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). The gel purified product was then PCR amplified using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc., Foster City, CA). The dye terminators were removed from the sequencing reaction mixture by passing the mixture through CENTRI-SEP columns (Princeton Separations, Inc., Adelphia, NJ) according to the manufacturer's instructions. Products were dried using a speed-vacuum and then submitted to the NCSU Sequencing Facility, where the samples were run on an ABI 377 automated sequencer.

Genetic Data Analysis

Tests for Hardy-Weinberg equilibrium and linkage disequilibrium

Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were analyzed by means of exact tests using the program Genetic Data Analysis ver. 1.1 (Lewis and Zaykin 2000; available via http://lewis.eeb.uconn.edu/lewishome/software.html) with 3200 iterations. Due to the strong family structure present in colonies (see Results), genotypes within colonies were not independent. Thus, only a single individual per colony was used for these tests. A resampling procedure was performed in which a single individual from each colony was selected at random for a total of 20 replications. The two transects at each location were pooled in the analyses.

Colony affiliations

Colony associations for the termites from each collection site were made based on microsatellite genotypes and mtDNA haplotypes. Each collection site was compared to all other collection points in the same transect. I compared the number of private alleles, that is, alleles unique to one group of workers in pairs of collection points. Collection points with the same alleles present and a consistent set of genotypes at all six of the microsatellite loci used in the final analyses (see Results) and with identical mtDNA haplotypes were considered to belong to the same colony. Finally, I compared estimates of worker relatedness between collection points with the relatedness coefficient among workers within collection points using the program Relatedness ver. 5.0.8 (Queller and Goodnight 1989; available via http://gsoftnet/GSoft.html). Relatively high coefficients of relatedness between workers from different collection points meeting the above criteria were taken as additional evidence for con-coloniality. In practice, the high variability of the markers and the close family structure of colonies (see Results) made colony designations unambiguous, and, with one exception (see Results), pairs of collection points within a transect contained on average 14 private alleles and did not show high relatedness between workers, indicating that nearly all collection points represented distinct colonies.

Classification of colonies

Based on the microsatellite genotypes present and their frequencies, colonies were classified as either simple families or extended families. Simple family colonies were those in which worker genotypes at all loci were consistent with being the direct offspring of monogamous pairs of reproductives and the observed frequencies of the genotypes did not differ significantly from expected frequencies as determined by a *G*-test. For each colony, all the locus-specific *G*-values were summed to obtain an overall *G*-value. Extended family colonies were those in which there were genotypes inconsistent with a single pair of reproductives at one or more loci (e.g., five or more genotypic classes or three classes of homozygotes) or those in which genotypes were consistent with simple families but their frequencies deviated significantly from expected ($P < 0.05$, *G*-test). Extended family colonies are those that are presumably headed by multiple neotenic reproductives by themselves or together with one or both of the primary reproductives.

Colony and population genetic structure

Colony and population genetic structure was investigated at several levels. To assess kin structure within colonies, I estimated the average relatedness for workers from the microsatellite genotype frequencies using the program Relatedness ver. 5.0.8 (Queller and Goodnight 1989) with colonies weighted equally. The 95% confidence intervals (CIs) were obtained by jackknifing over loci. Within-colony relatedness was corrected for genetic structure at the level of the transect using the demes option.

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 Genetic Data Analysis (Lewis and Zaykin 2000). I followed the notation of Thorne et al. (1999) and Bulmer et al. (2001), in which each colony is treated as a population and 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}. F_{CT} represents genetic differentiation among colonies and is similar to F_{ST}. The colony inbreeding coefficient, F_{IC}, has no analog in solitary organisms and is especially sensitive to the numbers of reproductives and their mating patterns within social groups. Specifically, F_{IC} is expected to be strongly negative in simple family colonies, approach zero with increasing numbers of reproductives, and become positive with assortative mating among multiple reproductives within colonies or with mixing of individuals from different colonies. In addition, I estimated F_{ST}-values among transects within sites as well as the overall F_{ST} among sites. The 95% CIs were constructed by bootstrapping over loci with 1000 replications. *F*-statistics were estimated for all colonies together, as well as for simple family colonies and extended family colonies separately. These values were then compared to values generated by Thorne et al. (1999) and E. Adams (unpubl. data) in computer simulations of possible breeding systems of *Reticulitermes* species. Values whose 95% CIs did not overlap were considered to be significantly different at the $\alpha = 0.05$ level. Genotypes of the reproductives in each simple family colony were inferred, except, of course, in the one colony in which the collected primary pair was genotyped directly. *F*-statistics and the average relatedness between the reproductives in these colonies were estimated from the reconstructed genotypes.

A neighbor-joining tree was constructed to further investigate the genetic relationships among transects using several programs within the package PHYLIP 3.6α3 (Felsenstein 1989; available via http://evolution.genetics.washington.edu/phylip.html). The allele frequencies obtained from all workers in the sampled transects were used to generate 1000 datasets by bootstrapping over loci using the SEQBOOT program. Next, Nei's (1972) genetic distance was calculated between all pairs of transects from each of the bootstrap replicates using GENDIST. The resulting distance matrices were then used to construct 1000 neighbor-joining trees using NEIGHBOR. Finally, a strict consensus tree with confidence levels for each of the nodes was constructed using the program CONSENSE.

Genetic structure at the level of the transect and site was also investigated by estimating Φ-statistics from mtDNA sequence data using the analysis of molecular variance (AMOVA) method of Excoffier et al. (1992) as implemented in the program ARLEQUIN (Raymond and Rousset 1995; available via http://lgb.unige.ch/arlequin/). These analyses were done on the Euclidian square distances between pairs of haplotypes according to the method of Tajima and Nei (1984). The significance probabilities for the mtDNA Φ-statistics were generated using permutation analysis on 10,000 randomly permuted distance matrices. A neighbor-joining tree showing the genetic relationships of the haplotypes from each site was constructed from the pairwise Euclidian distances using MEGA ver. 2.1 (Kumar et al. 2001; available via http://www.megasoftware.net/) and confidence levels for the nodes were obtained by bootstrapping 1000 times. Parsimony scores for the topology of the neighbor-joining tree were generated using PAUP*4.0b10 (Swofford 1998).

Isolation by distance analysis

Isolation by distance was assessed from the microsatellite genotypes by estimating the coefficient of relatedness (*r*) between workers from one colony and those in another colony for all pairs of colonies within a transect. The Pearson product correlation coefficient was then computed for the pairwise relatedness values and the physical distance for all colony pairs within a transect. Given the close family structure of colonies (see Results), the pairwise coefficient of relatedness was chosen rather than pairwise F_{ST}-values to investigate isolation by distance; the former method should give a more accurate measure of the genetic relationships among colonies because it takes into account the allele frequencies in the entire population (site), whereas the latter uses only the alleles present in the two colonies. Isolation by distance was also investigated using the mtDNA data by correlation analysis of the Euclidian distances of mtDNA haplotypes and the physical distance among all colony pairs within a transect. The significance of the correlation coefficients was assessed by means of a Mantel test with 1000 replications as implemented in Genepop on the Web ver. 3.1c (Raymond and Rousset 1995; available via http://wbiomed.curtin.edu.au/genepop/).

RESULTS

Basic Genetic Data

In total, 1272 individuals were genotyped at nine variable microsatellite loci. Close inspection of genotypes, especially from the family analysis described below, revealed a null allele at locus *Rf 3-1* (data not shown). This locus was therefore excluded in subsequent analyses. As shown in Table 1, there were four to 32 alleles per locus within the study population at the eight remaining loci.

Comparison of the observed genotypes to the expected for the colony in which the primary king and queen were collected confirmed Mendelian inheritance of all loci. All expected genotypic classes among the progeny of the king and queen were present among the workers, and the ratios of the genotypes at each locus did not differ significantly from expected ($G = 21.8$, df $= 20$, $P > 0.25$).

TABLE 1. Allele numbers and frequency of most common allele at eight microsatellite loci for the *Reticulitermes flavipes* study population.

Locus	No. alleles	Freq. most common allele
Rf 1-3	12	0.36
Rf 5-10	6	0.50
Rf 6-1	12	0.59
Rf 11-1	6	0.32
Rf 11-2	4	0.77
Rf 15-2	4	0.79
Rf 21-1	25	0.23
Rf 24-2	32	0.19
Mean	12.6	

Two loci, *Rf 11-2* and *Rf 21-1*, showed significant deviations from HWE in more than 50% of the 120 tests performed (20 resampled datasets × 3 populations × 2 loci), with deviations occurring frequently across all three populations. Such deviations may be due to selection at the loci or a scoring problem, such as the presence of null alleles. Although inclusion of these loci in the analyses had little effect on the results, they were excluded from the analyses presented here. The remaining six loci showed significant deviations from HWE in only 50 of 360 tests (14%; 20 resampled datasets × 3 populations × 6 loci), with no consistent pattern across loci or across populations. Results of the exact tests for linkage disequilibrium for pairs of the six loci showed significant linkage disequilibrium for 194 of the 900 locus/population combinations (22%), but again there were no consistent pattern across loci or populations. Thus, the six microsatellite loci used in the analyses were considered to be independently assorting markers conforming to HWE and suitable for colony and population genetic analysis. The mean number of alleles for these six loci was 9.0.

The two restriction enzymes cut the amplified mtDNA fragment at no, one, or two sites, yielding between one and three bands. Altogether, 14 haplotypes could be distinguished, with the most common haplotype occurring in 24 of the 56 colonies (43%). However, there was variation in the size of the amplified product (1600–2500 bp), thereby confounding the analysis of haplotypes based on restriction sites. Although the PCR-RFLP analysis of the AT-rich region proved useful in investigating haplotype numbers within colonies, sequence data from a 334-bp fragment containing 52 (15.6%) variable positions, of which 28 (8.4%) were parsimony informative, were used for examining population structure. A total 40 sequence haplotypes were found, of which 33 (82.5%) were unique, with the frequency of the most common haplotype being 0.20.

Grouping of Collection Points into Colonies

As described below, all workers within a collection point either belonged to simple families or extended (inbred) families derived from simple families. Consequently, workers from different collection points could confidently be excluded as belonging to the same colony based on the incompatibility of genotypes among the different collection points. Of the 57 collection points, only a single pair of adjacent collection points separated by 15 m and located in the Lake Wheeler site had compatible (and nearly identical) genotypes at all six microsatellite loci, and the genotypes in the two groups were present in similar frequencies (Table 2). The coefficient of relatedness (± SE) between workers in these groups was high ($r = 0.481 \pm 0.079$) and nearly identical to the values

TABLE 2. Genotypes of *Reticulitermes flavipes* worker groups ($n = 20$ for most loci but may be fewer in some cases) collected from the four consecutive sampling points in one of the Lake Wheeler transects. Sampling points 1 and 2 were from the same colony, whereas the other sampling points contained workers from different colonies.

Locus/ genotype	Sampling point 1[1]	Sampling point 2[1]	Sampling point 3[1]	Sampling point 4[2]
Rf 1-3				
218/218			1	
218/233			4	
227/227			1	19
227/233			5	
230/230	1	4		
230/236		1		
230/248	2	4		
236/236	3	1		
236/248	2	5		
Rf 5-10				
147/147			1	
147/153			7	
147/159			1	
153/153	11	11	3	11
153/159	5	6	4	8
153/162				
Rf 11-1				
241/249			3	
243/251				9
249/249	7	3	11	
249/251	2	6		
251/251	2	6		11
Rf 24-2				
158/164				12
158/182	2	3		
158/200	1	3		
164/203				8
182/182	4	4		
182/185			5	
182/194			7	
182/200	5	8		
185/194			3	
194/194			4	
Rf 15-2				
232/232			6	
232/235			8	8
235/235	11	17	2	11
Rf 6-1				
164/167				11
164/170				4
167/170			6	
167/176			3	
167/209				2
170/170	8	11		
170/173			5	
170/176	11	8		
170/209				3
173/176			5	

[1] Extended family colony.
[2] Simple family colony.

TABLE 3. Numbers of simple family and extended family colonies within each transect and site.

Site	Total no. colonies	No. simple family colonies	No. extended family colonies
Duke Forest			
Transect 1	10	7 (70.0%)	3 (30.0%)
Transect 2	10	8 (80.0%)	2 (20.0%)
Subtotal	20	15 (75.0%)	5 (25.0%)
Lake Wheeler			
Transect 1	7	4 (57.1%)	3 (42.9%)
Transect 2	10	7 (70.0%)	3 (30.0%)
Subtotal	17	11 (64.7%)	6 (35.3%)
Schenck Forest			
Transect 1	9	8 (88.9%)	1 (11.1%)
Transect 2	10	9 (90.0%)	1 (10.0%)
Subtotal	19	17 (89.5%)	2 (10.5%)
All sites combined	56	43 (76.8%)	13 (23.2%)

of relatedness among nestmate workers within each group ($r = 0.484 \pm 0.064$ and 0.490 ± 0.077). In addition, these two groups had identical mtDNA sequence haplotypes. Thus, these collection points were considered to be part of the same colony. In contrast, all other groups of workers from different collection points had very different genotypes. The average number of private alleles between groups of workers in pairs of collection points within a transect was 14.1 (range = 6–25; 93 comparisons), for a mean difference of 2.3 alleles per locus. Considering only the most polymorphic locus, *Rf 24-2*, worker groups from all pairs of collection points within a transect differed on average by 5.3 alleles.

Typical differences in the allele and genotype compositions of different colonies are shown in Table 2. In this example, sampling points 1 and 2 were considered to belong to the same colony, because they had the same alleles present at every locus and had all the same genotypes except at *Rf 1-3*, in which there was one individual in sampling point 2 that had a unique genotype (*230/236*). However, this genotype was compatible with the set of alleles present in sampling point 1. In contrast, the three separate colonies shown in Table 2 differed at several loci, especially *Rf 24–2* and *Rf 6-1*, in which there were several private alleles and no genotypes shared in common.

Finally, whereas the average relatedness of workers within collection points was high ($r = 0.503 \pm 0.024$), average relatedness between all pairs of adjacent collection points within each of the six transects (excluding the one pair of adjacent collection points grouped into the same colony) was not significantly different from zero ($r = -0.019 \pm 0.116$, range = -0.382 to 0.315), and there were no pairs of colonies in which the workers were significantly related. Thus, based on these analyses, workers from the 57 collection points were considered to belong to 56 different colonies.

Once collection sites were identified to colony, I then calculated the distance among neighboring colonies that were sampled. Overall, the average (± SD) distance between neighboring colonies was 25.4 ± 11.1 m. There was some variation among sites; the Schenck Forest and Duke Forest sites were similar (22.6 ± 9.4 and 22.0 ± 7.0, respectively), whereas colonies were more dispersed at the Lake Wheeler site (32.9 ± 14.2). There was a significant difference among sites ($F_{2,46} = 5.21$, $P < 0.01$), with the distances among neighboring colonies being significantly greater at the Lake Wheeler site than at the other two sites ($P < 0.05$, Tukey test), suggesting that colonies were less abundant at the Lake Wheeler site.

Classification of Colonies

As suggested by the high level of nestmate relatedness, all colonies formed close family units. Despite the high variability of the microsatellite markers, no group of nestmate workers had more than four alleles at a locus, the maximum number of alleles possible in colonies comprised of individuals descended from a single pair of founders. This conclusion is supported by the mtDNA data showing only a single haplotype present in each colony, suggesting that all workers within a colony are derived from a single pair of founding alates.

As shown in Table 3, the majority of colonies were simple families, ranging from about 65% to 90% across sites. The two transects within each site were very similar in the proportion of simple family colonies. Overall, 43 of the 56 colonies (76.8%) were simple families. In nearly all cases, extended family colonies had more genotypes than could be produced by a single pair of reproductives, most likely due to the presence of neotenic reproductives derived from the founding king and queen.

Inference of Breeding System

The values for the *F*-statistics and relatedness coefficient are shown in Table 4, as well as values expected for different breeding systems as derived by computer simulations by Thorne et al. (1999), Bulmer et al. (2001), and E. Adams (unpubl. data). For all colonies combined, nestmate workers were closely related, with a relatedness coefficient equivalent to that expected for full-sibs ($r = 0.5$). Workers were, on average, somewhat inbred relative to the total population ($F_{IT} = 0.091$). However, compared to simple family colonies, higher values for all the *F*-statistics in the extended family colonies are consistent with greater levels of inbreeding in

TABLE 4. *F*-statistics and relatedness coefficients for worker nestmates of *Reticulitermes flavipes* from three study sites. Also included are values expected for several possible breeding systems as derived from computer simulations by Thorne et al. (1999), Bulmer et al. (2001), and E. Adams (unpubl. data). For the simulated breeding systems, X represents the number of generations of production of replacement reproductives within a colony; N_f and N_m represent the number of replacement females and males, respectively, produced per generation. Values in parentheses are 95% CIs.

Site	F_{IT}	F_{CT}	F_{IC}	r
All sites combined				
All colonies ($n = 56$)	0.091 (0.008 to 0.184)	0.311 (0.253 to 0.381)	−0.319 (−0.351 to −0.261)	0.515 (0.433 to 0.597)
Simple family colonies ($n = 43$)	0.062 (−0.005 to 0.142)	0.305 (0.247 to 0.372)	−0.349 (−0.388 to −0.286)	0.517 (0.424 to 0.610)
Extended family colonies ($n = 13$)	0.207 (0.043 to 0.363)	0.340 (0.243 to 0.439)	−0.201 (−0.264 to −0.125)	0.508 (0.446 to 0.570)
Duke Forest				
All colonies ($n = 20$)	0.026 (−0.044 to 0.107)	0.269 (0.226 to 0.306)	−0.333 (−0.394 to −0.259)	0.522 (0.430 to 0.613)
Simple family colonies ($n = 15$)	0.006 (−0.061 to 0.084)	0.266 (0.221 to 0.299)	−0.354 (−0.432 to −0.275)	0.526 (0.432 to 0.620)
Extended family colonies ($n = 5$)	0.089 (−0.036 to 0.241)	0.281 (0.205 to 0.341)	−0.267 (−0.385 to −0.177)	0.510 (0.422 to 0.598)
Lake Wheeler				
All colonies ($n = 17$)	0.122 (0.028 to 0.237)	0.307 (0.246 to 0.379)	−0.268 (−0.294 to −0.233)	0.549 (0.428 to 0.671)
Simple family colonies ($n = 11$)	0.095 (0.004 to 0.231)	0.317 (0.245 to 0.414)	−0.325 (−0.343 to −0.301)	0.571 (0.415 to 0.728)
Extended family colonies ($n = 6$)	0.158 (0.021 to 0.284)	0.279 (0.212 to 0.342)	−0.167 (−0.248 to −0.085)	0.512 (0.443 to 0.581)
Schenck Forest				
All colonies ($n = 19$)	−0.014 (−0.105 to 0.061)	0.243 (0.190 to 0.285)	−0.339 (−0.381 to −0.260)	0.474 (0.379 to 0.569)
Simple family colonies ($n = 17$)	−0.029 (−0.114 to 0.046)	0.241 (0.191 to 0.279)	−0.355 (−0.403 to −0.268)	0.471 (0.371 to 0.571)
Extended family colonies ($n = 2$)	0.127 (−0.010 to 0.226)	0.229 (0.113 to 0.312)	−0.132 (−0.200 to −0.067)	0.491 (0.301 to 0.681)
Simulated breeding system				
(A) Colonies headed by outbred reproductive pairs	0.00	0.25	−0.33	0.50
(B) Colonies with inbreeding among neotenics				
(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	0.78
(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
(C) Population of 75% simple families and 25% extended families				
(1) Colonies headed by outbred reproductive pairs	0.00	0.27	−0.36	0.53
(2) Colonies with inbreeding among neotenics				
(i) $N_f = 2$, $N_m = 1$, $X = 1$	0.26	0.35	−0.14	0.55
(ii) $N_f = N_m = 2$, $X = 1$	0.26	0.32	−0.09	0.51
(iii) $N_f = 5$, $N_m = 1$, $X = 1$	0.27	0.34	−0.11	0.53
(iv) $N_f = N_m = 5$, $X = 1$	0.27	0.29	−0.03	0.46
(3) Simple family and extended family colonies ($N_f = 2$, $N_m = 1$, $X = 1$) combined	0.07	0.29	−0.31	0.54

TABLE 5. *F*-statistics and relatedness coefficients (95% CI) for the reproductives ($n = 84$) in simple family colonies of *Reticulitermes flavipes* from three study sites. The genotypes of the reproductives were inferred from the genotypes of the worker offspring. The relatedness coefficient represents the degree of relatedness between reproductives within colonies.

F_{IT}	F_{ST} (among sites)	F_{ST} (between transects within sites)	F_{IS}	r
0.095 (−0.029 to 0.221)	0.055 (0.014 to 0.114)	0.060 (0.018 to 0.123)	0.037 (−0.071 to 0.129)	0.047 (−0.095 to 0.188)

the latter. The values estimated for simple family colonies are nearly identical to those expected for a population of colonies headed by monogamous pairs of outbred reproductives (Table 4, case A). As seen in Table 4, case C2, the most pronounced effect of increasing the number of neotenics within colonies is to push F_{IC}, the coefficient of inbreeding in individuals relative to their colony, from highly negative values toward zero (see also Thorne et al. 1999; Bulmer et al. 2001). The empirical values for the extended family colonies closely match the average values expected for colonies headed by two female and one male neotenic (case C2i). In contrast, all other modeled breeding systems can be ruled out because in every case the expected F_{IC} was significantly less negative than the empirically derived value for the extended family colonies. When both simple family and extended family colonies were pooled in the simulations (case C3), the expected values matched very closely the empirical values for all colonies combined, suggesting that the study population was a 3:1 mixture of simple families headed by outbred parents and extended families headed by a few sibling neotenics who were the direct progeny of the founding pair.

There was some variation among study sites in the *F*-statistics and relatedness coefficient (Table 4). Overall, workers in Schenck Forest were the least inbred and those in Lake Wheeler were the most inbred. Within each population, individuals in simple family colonies were slightly less inbred than individuals in extended family colonies, as evidenced by higher F_{IT}-values, although these differences were not significant for any of the three sites.

Results from the inferred and observed genotypes of the reproductives in simple family colonies (Table 5) suggest that the reproductives in these colonies are outbred, supporting the conclusion drawn from the analysis of worker genotypes. The coefficient of relatedness between the two reproductives in each colony was low and not significantly different from zero. The F_{IT}-value for reproductives was nearly identical to that for workers as a whole ($F_{IT} = 0.095$ and 0.091, respectively) and did not differ significantly from zero. There was significant genetic differentiation at both the transect and site levels, with values nearly identical to those among workers (transect level: $F_{ST} = 0.060$ and 0.065; site level: $F_{ST} = 0.055$ and 0.057, for reproductives and workers, respectively; Table 6).

TABLE 6. F_{ST}-values (95% CI) between transects within sites and among sites estimated from worker genotypes.

Level	F_{ST}
Between transects within sites	
All sites combined	0.065 (0.023 to 0.133)
Duke Forest	0.004 (−0.017 to 0.044)
Lake Wheeler	0.001 (−0.018 to 0.015)
Schenck Forest	0.016 (0.006 to 0.033)
Among sites	0.057 (0.009 to 0.131)

Genetic Differentiation within and among Sites

Estimates of genetic differentiation using the microsatellite data showed slight but significant differentiation both among sites and between transects within sites in the hierarchical analysis (Table 6). However, when sites were analyzed separately, there was significant differentiation (95% CIs not overlapping zero) only between transects at Schenck Forest. As shown in Table 7, results of AMOVA (Excoffier et al. 1992) indicated that nearly 80% of the total mtDNA haplotype variation was due to variation within sites, whereas variation among sites accounted for nearly 20%, with only 2% of the variation occurring between transects within sites, although these latter two values were not significant.

There was no evidence of isolation by distance in any of the transects, as would be expected if there was strong population viscosity due to limited dispersal of both male and female reproductives, either through budding or short-range mating flights. As seen in Figure 1, there was no significant correlation between relatedness of workers in pairs of colonies and the physical distance between the colonies in any of the transects (range of $r = -0.30$ to 0.37, all $P \geq 0.28$, Mantel test). Analyses using pairwise values of F_{ST}, $F_{ST}/(1 - F_{ST})$, and distance or ln(distance) gave essentially the same results (data not shown). Consistent with the lack of isolation by distance with the microsatellite data, the mtDNA sequence data also showed no significant positive relationship between pairwise differences in the number of nucleotide substitutions according to the Tajima and Nei (1984) distance method and geographic distance between colonies (Fig. 2; range of $r = -0.25$ to 0.45, all $P \geq 0.18$, Mantel test).

Because most colonies were simple families headed by outbred parents, population viscosity due to budding could be swamped out in the above analysis. Colonies originating from budding events are likely to be headed by neotenics that develop within the colony following budding. Thus, if budding is a common mode of reproduction, the resulting daughter colonies should be extended families that are closely related to the nearby parent colony from which they originated. In contrast to the predicted close relationship among neighboring colonies that would be expected if extended fam-

TABLE 7. Hierarchical analysis of variance of mitochondrial DNA sequence data (AT-rich region) using analysis of molecular variance (Excoffier et al. 1992).

	df	Variance components	Percent of total variation	*P*	Φ-statistics
Among sites	2	V_a	20.0	0.11	$\Phi_{CT} = 0.200$
Among transects within sites	3	V_b	1.9	0.18	$\Phi_{SC} = 0.024$
Within transects	50	V_c	78.1	<0.001	$\Phi_{ST} = 0.219$

ily colonies commonly arose through budding, relatedness between workers in extended family colonies and their nearest neighbor was not significantly greater than zero ($r = -0.072 \pm 0.099$). Again, the mtDNA sequence data gave a similar result: of the 13 extended family colonies, only one colony had the same haplotype as its nearest neighbor, providing further evidence that budding is not a frequent mode of colony reproduction in the study population.

A tree showing the genetic relationships among the mtDNA haplotypes is shown in Figure 3. Most of the Duke Forest haplotypes formed a distinct clade with little overlap between the Schenck Forest and Lake Wheeler haplotypes. However, the Lake Wheeler and Schenck Forest haplotypes were not obviously differentiated, and in fact shared one haplotype in common. In general, there was only weak bootstrap support for most of the nodes, especially the outer nodes, with the conspicuous exception of two haplotypes, one each from the Schenck Forest and the Lake Wheeler sites, which clearly separated from the other 38 haplotypes. These results, indicating no significant haplotype differentiation among the three sites, are in agreement with the results of the AMOVA, in which nearly all the variation was within transects. The separation of the Duke Forest haplotypes from the those of the other two sites is consistent with the geographic distance among the sites, with Duke Forest separated from the Schenck Forest and Lake Wheeler sites by about twice the distance (27 km and 37.6 km, respectively) separating the Schenck Forest and Lake Wheeler sites (15.9 km). A tree showing the genetic relationships among the transects based on the microsatellite data is shown in Figure 4. Again, the Duke Forest transects show close affinities, forming a distinct, well-supported clade. The Schenck Forest transects also show close affinity to each other, whereas the Lake Wheeler transects are slightly diverged, with transect 1 being somewhat more allied with the Schenck Forest transects.

DISCUSSION

Colony Breeding Structure

These results show that colonies in the study population were comprised of close family units that were initiated by monogamous pairs of unrelated reproductives. The majority of colonies were simple families headed by the founding king and queen. The remainder of the colonies had worker genotypes consistent with inbred (extended) families derived from simple families. The extended family colonies, which were presumably headed by multiple neotenics, showed signs of only moderate inbreeding, indicative of colonies with low numbers of reproductives that are the direct progeny of the original king and queen (Table 4, case C2i). The close match between the empirically derived values for the coefficients of relatedness and inbreeding and those expected from computer simulations suggest that a model of a 3:1 mixture of simple families and extended families headed by two female and one male neotenic provides a reasonable approximation for the breeding system of this population.

Although there was some variation among sites in the proportions of simple family and extended family colonies, ranging from about 65% simple families in the Lake Wheeler site to nearly 90% in the Schenck Forest site, the values of the *F*-statistics and nestmate relatedness for colonies were very similar across sites, suggesting that the basic breeding structure for each colony type was fairly uniform throughout the study area. The observed variation in frequency of colony type is likely due to differences among sites in colony age structure. Simple family colonies headed by outbred reproductives, such as those in the study population, are expected to be younger than extended family colonies because the production of neotenics within colonies should occur later in colony life, after one or both of the original primary reproductives dies (Thorne 1998; Thorne et al. 1999). To the extent that this is true, most of the study colonies were relatively young. An age structure biased toward young colonies could arise if most colonies in the study area were of similar age, having been founded concurrently, perhaps with the sudden availability of suitable habitat and/or food resources. It seems unlikely that new, previously unavailable nest sites opened up more or less simultaneously within all six transects. However, it should be noted that in September 1996 Hurricane Fran felled many trees across the region, resulting in large quantities of decaying wood. Thus, it is possible that this sudden influx of wood resources might have permitted the establishment of many new colonies, resulting in an atypical age distribution when the sites were sampled in 1999. An alternative explanation for the prevalence of simple family colonies is that most colonies within the study area do not live long enough to produce neotenics. Recent results from an urban habitat in Raleigh, North Carolina, where there was no large addition of dead wood in 1996, show very similar proportions of simple family colonies (73%; Vargo 2003) suggesting that the age structure of the study population may be typical for this area and was not appreciably altered by Hurricane Fran. It is interesting to note that the Lake Wheeler site had the highest frequency of the presumably older extended family colonies and the lowest density of colonies, suggesting that establishment of new colonies at this site may be more difficult than at the other two sites.

Although reports of functional reproductives found in the field are rare, there is some evidence suggesting that the proportions of simple family colonies found in the present study may be typical for *R. flavipes* in the mid-Atlantic region. Over a five-year period, Banks and Snyder (1920) found re-

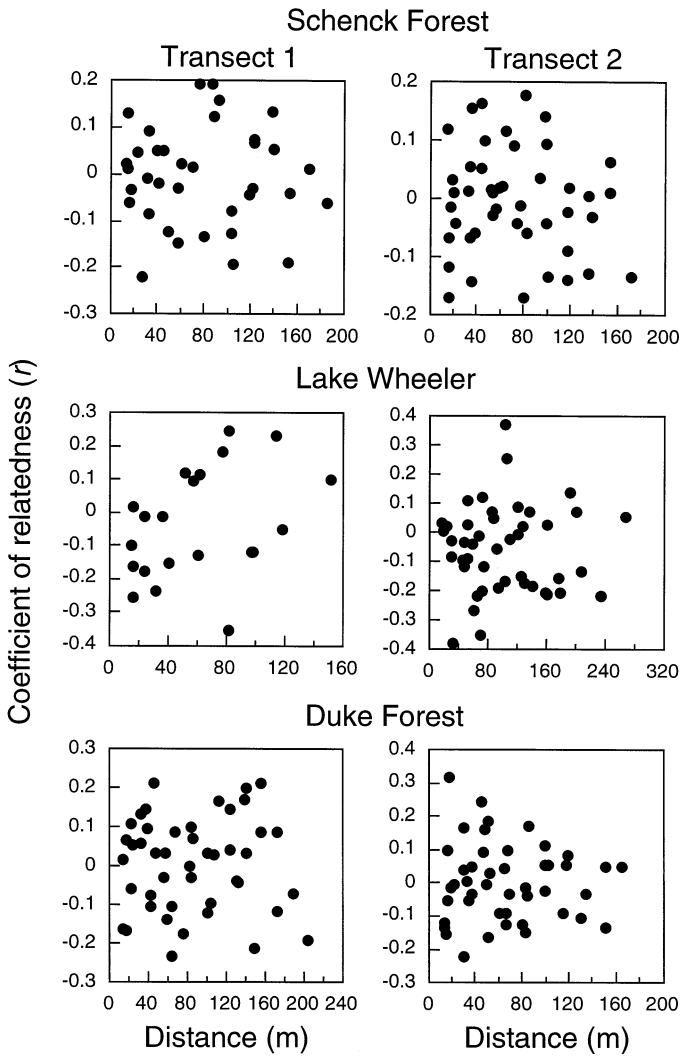

FIG. 1. Isolation-by-distance analysis for *Reticulitermes flavipes* colonies within transects using microsatellite data. The relationship between pairwise coefficients of relatedness among non-colonymates and geographic distance is shown. The correlation coefficients were not significant for any of the transects (range of $r = -0.30$ to 0.37; all $P \geq 0.28$, Mantel test).

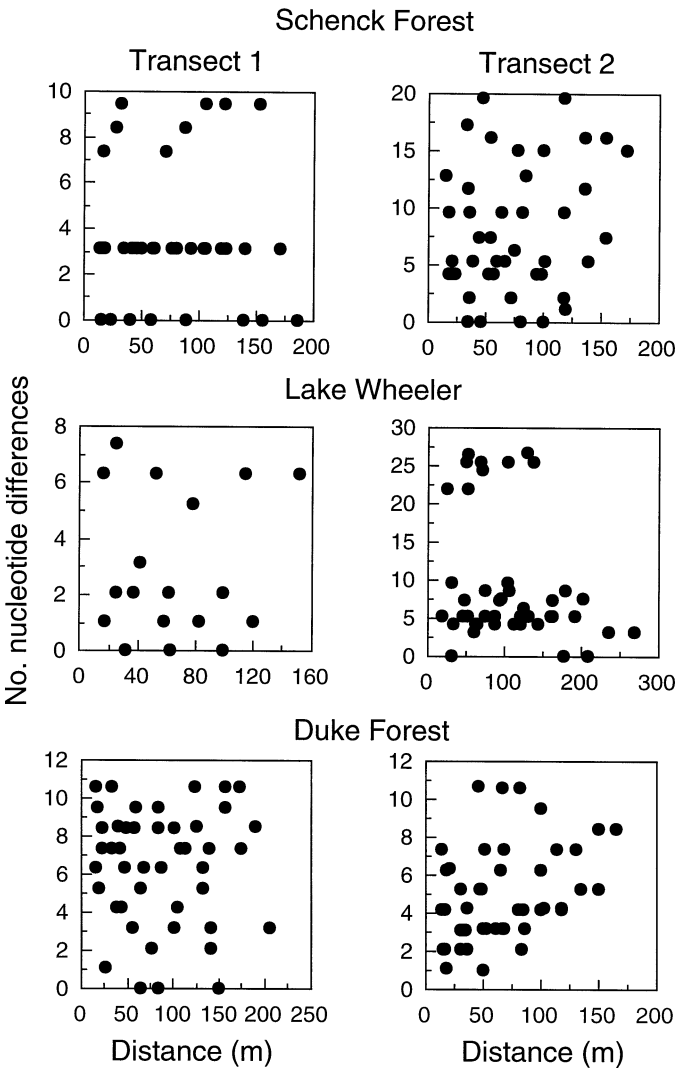

FIG. 2. Relationship between the pairwise number of nucleotide substitutions according to the Tajima and Nei (1984) method and geographic distance between *Reticulitermes flavipes* colonies within transects. The correlation coefficients were not significant for any of the transects (range of $r = -0.25$ to 0.45; all $P \geq 0.18$, Mantel test).

productives in 16 logs and stumps in northern Virginia, some 400 km north of the present study population. Primary reproductives were found in 12 (75%) cases, whereas neotenics were found in four, a 3:1 ratio of simple family to extended family colonies very similar to that found in the present study. The numbers of neotenics recovered by Banks and Snyder (1920) ranged from six to 29 females and zero to 15 males, which are higher numbers than the present data suggested occurred in the extended family colonies studied in North Carolina. Although the strongly negative F_{IC}-value found here indicates a low effective number of neotenics in extended family colonies, it is possible that there are many neotenics present in colonies but their effective number is low due to unequal production of offspring. Such reproductive skew is common among social insects (Reeve and Keller 2001) and it is an important component of their breeding systems (Ross 1993, 2001). However, reproductive skew has received little attention in studies of termite reproductive biology, so that its prevalence in the Isoptera is presently unknown. Future studies combining genetic data with detailed colony excavations and censuses of nest contents are needed to determine the extent, if any, of reproductive skew among neotenics in termite colonies.

The breeding system of the present study population differs to some extent from those of previous studies of *R. flavipes* from other areas as inferred from allozyme data. In a recent study near the northern edge of the range of this species, Bulmer et al. (2001) found variation in colony genetic structure between two sites separated by 0.5 km in Massachusetts. Simple family colonies comprised 37.5% (six of 16) of the colonies at one site, and the coefficient of relatedness and *F*-statistics indicated that these colonies were headed by outbred reproductives as in the present study. The remainder of the colonies at this site were inbred, with statistics corresponding

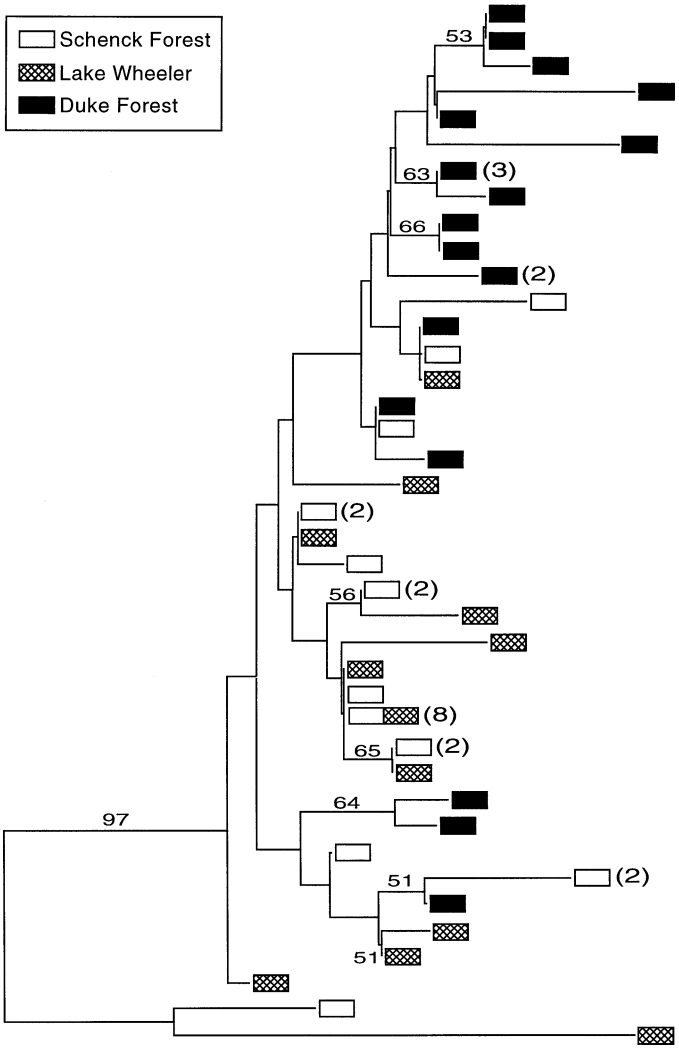

FIG. 3. A neighbor-joining tree showing the genetic relationships among mitochondrial DNA haplotypes found in the three *Reticulitermes flavipes* sites based on partial sequence of the AT-rich region. Numbers on parentheses indicate the frequency of nonunique haplotypes. Bootstrap values (percentage out of 1000 replicates) are given for nodes with greater than 50% support. Branch lengths depict genetic distance between nodes. Tree length was 89, with a consistency index of 0.685 and a retention index of 0.804.

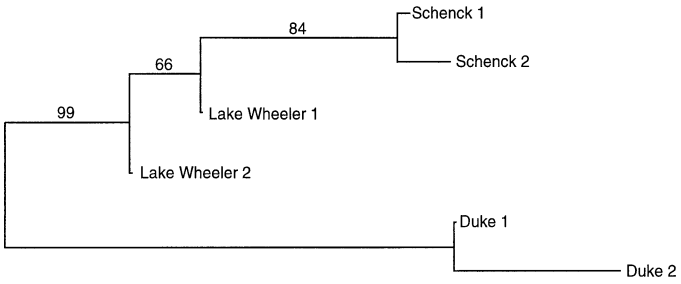

FIG. 4. Genetic relationships among *Reticulitermes flavipes* transects based on microsatellite data. The percentage of bootstraps out of 1000 replicates is indicated for nodes with greater than 50% support. Branch lengths are proportional to the genetic distance between nodes.

to colonies with tens or hundreds of neotenics inbred for several generations, considerably greater levels of inbreeding than that found in the present study. At the second site, Bulmer et al. (2001) found six colonies, which had more expansive foraging ranges and were on average highly inbred (F_{IT} = 0.34). These colonies had positive F_{IC}-values, from which the authors concluded that there was some commingling of workers from different colonies. Bulmer et al. (2001) attributed the differences in colony genetic structure between the two sites to variation in age structure and/or soil conditions that may influence the tunneling ability of termites and therefore the likelihood that foragers will encounter neighboring colonies with which they may merge. In Tennessee, Reilly (1987) found that colonies were, on average, much more inbred than those in either the present study (F_{IT} = 0.62 compared to 0.09) or the two sites studied by Bulmer et al. (2001; F_{IT} = 0.27 and 0.34), and had higher values for the coefficient of inbreeding in individuals relative to the colony (F_{IC} = 0.26 compared to −0.32 in the present study and 0.05 and 0.16 in Bulmer et al. 2001), suggestive of extensive commingling of workers from unrelated nests or frequent nest budding with interconnected daughter nests (Thorne et al. 1999; Bulmer et al. 2001). Unfortunately, Reilly (1986, 1987) did not provide information on the family structure of individual colonies, so that the frequencies of simple family and extended family colonies in the Tennessee study population are unknown. Thus, it seems that the breeding system of *R. flavipes* is labile and can vary considerably with geographic area as well as locally, perhaps in response to different ecological conditions.

The higher frequency of simple family colonies and lower levels of inbreeding found in the present study compared with the previous studies of *R. flavipes* conducted in central Tennessee (Reilly 1987) and Massachusetts (Bulmer et al. 2001) suggest that colonies in the Piedmont of North Carolina are shorter lived than those at the other sites. Perhaps the colder climate and/or other ecological factors in central Tennessee and Massachusetts make colony founding more difficult, selecting for longer-lived colonies that undergo several generations of inbreeding. Alternatively, there may be stronger selection against inbred colonies in the Piedmont of North Carolina.

Variation in *Reticulitermes* breeding systems has been found within and among four European species (Clément 1981, 1984, 1986; Clément et al. 2001). Clément (1986) proposed that in moister habitats, where there is abundant wood resources colonies are ''open,'' and there is free mixing of neighboring colonies to form large genetically complex groups, whereas in drier habitats with more limited food resources, colonies are small and far apart, resulting in infrequent encounters between neighboring colonies. Under the latter conditions, colonies form close family units. To the extent that the above characterization of breeding system in European *Reticulitermes* species is accurate, these species respond differently to local environmental conditions than does *R. flavipes*, because in the present study, colonies occurring in a moist forest habitat with an abundance of decaying wood were genetically distinct family groups with localized foraging areas. Variation in breeding systems has been reported for other termite species as well. For example,

Goodisman and Crozier (2002) found differences in the levels of inbreeding among three populations of the primitive termite *Mastotermes darwiniensis* (Mastotermitidae) in Australia. Thus, geographic and/or local variation in breeding systems may be widespread in termites.

Isolation by Distance

Colony reproduction by budding is believed to be common in multiple site-nesting termites, which include many subterranean species (reviewed in Shellman-Reeve 1997; Myles 1999; Thorne et al. 1999). Although there is circumstantial evidence that budding occurs in at least some populations of *Reticulitermes* (Thorne et al. 1999), conclusive evidence demonstrating budding in any population is lacking. Results of isolation by distance analysis using both the microsatellite and mtDNA markers in the present study suggest that there is no appreciable population viscosity on a local scale, as would be expected if reproduction by budding or very short-range mating flights occurred frequently in the study population. This conclusion is supported by the absence of significant relatedness between the extended family colonies and their nearest neighbors. The apparent lack of budding is consistent with the high proportion of simple family colonies headed by outbred primary reproductives. The process of budding would likely produce daughter colonies headed by neotenics. Therefore, in populations where budding is common, one would expect a relatively high proportion of extended family colonies headed by neotenics. It should be noted that conclusions drawn from the present results are tentative because the sampling regime used was not of a sufficiently fine scale to ensure that each colony's nearest neighbors were sampled; daughter colonies, if they existed, could have been missed. However, in a finer-scale study conducted in Massachusetts, Bulmer et al. (2001) also did not find evidence for frequent colony reproduction by budding in *R. flavipes*. In summary, although budding may occur often in some populations of *Reticulitermes*, especially populations with relatively high frequencies of extended family colonies, results to date from genetic data suggest that budding is not a common mode of reproduction in all populations and may not be as prevalent as was previously believed.

Results from other multiple-site nesting termites suggest budding may be an important mode of reproduction in some species but not others. Isolation-by-distance analysis on local scales of up to 1 km suggests frequent reproduction by budding in the African subterranean termite *Schedorhinotermes lamanianus* (Husseneder et al. 1998), but not in the primitive termite *M. darwiniensis* (Mastotermitidae) in northern Australia (Goodisman and Crozier 2002). Many more fine-scale genetic studies are needed to determine the frequency of budding in different species, the extent to which budding varies within species, and the role of ecological factors in promoting budding.

Size of Foraging Areas

Reticulitermes species are generally believed to form large, expansive colonies (Thorne 1998). There is evidence, primarily from mark-release-recapture studies in disturbed, urban areas that colonies of *R. flavipes* can occupy large foraging areas, extending up to 70 linear meters (Grace et al. 1989; Su et al. 1993). The present results suggest that *R. flavipes* colonies in the study area are fairly localized with foraging ranges that generally do not exceed 30 linear meters. Of the 56 colonies sampled, only one occupied more than a single collection point, and the two collection points spanned by this colony were separated by 15 m. Indeed, ongoing finer-scale studies of a longitudinal nature in two of the three sites studied here (DeHeer and Vargo 2004) also show fairly localized foraging ranges of *R. flavipes*. Additional evidence for limited foraging areas in this species comes from a detailed study by Bulmer et al. (2001) conducted in Massachusetts. Of the 22 colonies studied, 20 colonies had foraging ranges less than 20 linear meters, whereas the remaining two colonies extended approximately 65 and 76 linear meters. In sum, although there have been a few reports of expansive foraging ranges in *R. flavipes*, the above-mentioned studies as well as additional studies on this and other *Reticulitermes* species suggest that relatively localized colonies may be typical in this genus (Forschler and Ryder 1996; Tsunoda et al. 1999; Haverty et al. 2000).

Higher-Level Genetic Structure

Analyses of higher-level population structure using the microsatellite markers indicated weak but significant differentiation at both the level of the transect, a distance of 1 km, and the level of the site, a distance of 16–38 km. These results suggest that reproductives normally disperse over fairly large areas with small barriers to gene flow at these spatial scales. Although no significant structure was detected with the mtDNA data, the neighbor-joining tree (Fig. 3) suggested that the majority of Duke Forest haplotypes were closely allied and distinct from those at the other sites, whereas the haplotypes of the geographically closer Schenck Forest and Lake Wheeler sites were interspersed with each other. The lack of statistically significant structure in the mtDNA genome may be due to the smaller sample size used with this class of markers, which was effectively limited to the number of colonies. The weak differentiation at the microsatellite loci and lack of detectable structure in the mtDNA marker suggest that males and females do not exhibit major differences in dispersal distances.

The detection of structure among populations using microsatellites in the present study contrasts with the results of previous studies of *R. flavipes* employing allozymes at similar spatial scales; Reilly (1986, 1987) failed to detect significant differentiation among sites separated by 0.8 km or 10–100 km apart in Tennessee, and Bulmer et al. (2001) did not detect significant structure between sites separated by 0.5 km or by 20 km in Massachusetts. The differences between the present results and those of the previous studies could be due to the tighter confidence intervals resulting from the larger sample sizes used in the present study, the greater sensitivity of highly polymorphic microsatellites to detect differentiation compared to allozymes (e.g., Estoup et al. 1998; Ross et al. 1999), or differences in dispersal distances and gene flow in these geographic regions.

Although there was significant structure detected in the present study, the magnitude of differentiation was weak.

Thus, taken as a whole, the population genetic studies on *R. flavipes* indicate that there is only weak differentiation, at best, among sites separated by 0.5–100 km. At larger scales, Reilly (1986) reported significant differentiation at allozyme loci among sites in the southeastern United States separated by 100–600 km (F_{ST} = 0.12, 95% CI = 0.05–0.19), but Jenkins et al. (1999a) found no obvious relationship between mtDNA haplotype and geographic region across the state of Georgia. It therefore seems that there is extensive gene flow in *R. flavipes*, even at large spatial scales, and that this could constrain the degree to which components of the breeding system may be genetically adapted to local ecological conditions. If so, much of the observed variation in the breeding system of this species may be due to phenotypic plasticity in response to local ecological factors and demographic structure rather than adaptive genetic variation. A major challenge for future work in this area will be to assess the degree to which breeding system components in *R. flavipes* may respond to local selection and to investigate the effect of gene flow on adaptation to specific habitats. A variety of additional studies will be key in meeting this challenge, including characterization of the breeding system in populations across a wide geographic area, identification of specific ecological factors associated with breeding system variation in different habitats, and evaluation of the extent and direction of gene flow among populations.

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