MOLECULAR GENETIC ANALYSIS of COLONY and POPULATION GENETIC STRUCTURE of the EASTERN SUBTERRANEAN TERMITE *RETICULITERMES FLAVIPES* (ISOPTERA: RHINOTERMITIDAE) in NORTH CAROLINA

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Abstract To investigate the social organization of *Reticulitermes flavipes*, we studied three populations in undisturbed wooded areas of North Carolina - one in each of the Piedmont, the Blue Ridge Mountains and the Coastal Plain regions. We collected workers from natural wood debris, genotyped 20 workers per colony at eight microsatellite loci, and obtained mitochondrial DNA sequence data for individuals from each colony in the Piedmont population. We estimated the coefficient of relatedness (*r*) and *F*-statistics to infer basic features of social organization. Analysis of the genotypes within colonies showed that 32 (36%) of the 90 colonies were simple (Mendelian) families, and estimates of *r* and *F*-statistics suggest that these Mendelian colonies are headed by single pairs of outbred reproductives in the Coastal Plain and Piedmont sites, while colonies in the Blue Ridge Mountains site showed some signs of inbreeding. With one exception, the remaining 58 (64%) colonies appeared to have been founded by single pairs of reproductives with some level of inbreeding developing subsequently, presumably through the production of neotenic reproductives. Values of *r* and *F*-statistics for these non-Mendelian colonies suggest that they contain only a few neotenic reproductives inbred for only a couple of generations. The one exceptional colony appeared to have multiple unrelated reproductives.

Key Words Microsatellites mitochondrial DNA markers breeding structure social organization gene flow

INTRODUCTION

Detailed information on subterranean termite social organization is critical to the development and implementation of more effective methods of control, especially with the growing interest in baiting technologies. Yet social organization within colonies of subterranean termites (Rhinotermitidae), including *Reticulitermes* spp., remains poorly known, largely because their cryptic nesting and foraging habits make it difficult to find the reproductives. Nevertheless, we have a general understanding of subterranean termite life history and the different types of social organizations they can form (reviewed in Thorne, 1998; Thorne et al., 1999). Colonies are generally thought to be founded by a single pair of primary reproductives following their nuptial flight. Early on, colonies thus consist of simple families headed by a single pair of primary reproductives. However, later in colony life, a number of events can lead to complex social structures.

Over time, one or both of the primary reproductives may be replaced by neotenic reproductives that remain in the colony and inbreed. The capacity to generate large numbers of neotenic reproductives is a unique feature of many subterranean termites, especially *Reticulitermes* spp., that is believed (see e.g., Thorne, 1998) to be responsible for producing the very populous and expansive colonies often associated with this group.

Colonies that reach large population sizes may form expansive underground networks consisting of several interconnected nests and foraging sites. It is possible that many of these large colonies will fragment, with some population centers becoming disconnected and independent of the others, leading to colony reproduction by budding. Furthermore, it is possible that unrelated

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colonies can fuse, or that unrelated reproductives are recruited into established colonies (Clément, 1981; Jenkins et al., 1999a; Bulmer et al., 2001; Matsuura and Nishida, 2001).

Thus a population of *Reticulitermes* spp. can consist of a complex mixture of colonies that differ in their social organizations. Some colonies within a population may be comprised of simple family units. Others in the same population can have numerous inbred neotenic reproductives, some of which may split off to form independent colonies. Still other colonies may represent fused remnants of unrelated colonies. The relative frequencies of these types of colonies collectively constitute a population's breeding system, and knowledge of this breeding system is pivotal to understanding how and under what conditions both current and future termite control measures might be effective.

The most practical way to test hypotheses about the breeding system of a social insect population is to examine the distribution of genotypes within and among colonies (Ross, 2001). The use of genetic markers to examine colony and population structure in termites is gaining interest (e.g., Luykx, 1985; Korman and Pashley, 1991; Korman et al., 1991; Strong and Grace, 1993; Kaib et al., 1996; Atkinson and Adams, 1997; Husseneder et al., 1998, 1999; Thompson and Hebert, 1998a, b; Thompson et al., 2000; Bulmer et al., 2001; Husseneder and Grace, 2001; Goodisman and Crozier, 2002). A major conclusion from this work is that there is considerable variability in breeding systems, both within and among various termite species.

To date there has been only a handful of population genetic studies on *Reticulitermes* spp. (Clément 1981, 1984; Reilly, 1987; Jenkins et al., 1999a, b; Bulmer et al., 2001), and only three of these (Clément, 1981; Reilly, 1987; Bulmer et al., 2001) directly addressed colony and population structure. The most powerful approach to investigate breeding systems of social insects is to combine pedigree analysis of individual colonies with population-wide measures of genetic structure, mainly the coefficient of relatedness and *F*-statistics (Ross, 2001). The study by Bulmer et al. (2001) on a population of *R. flavipes* in Massachusetts is the sole investigation to date to apply this method in *Reticulitermes*. The main objective of this study was to use molecular genetic markers to characterize the breeding system in each of three populations of the eastern subterranean termite, *R. flavipes*, one population from each of the three main regions of North Carolina.

MATERIALS and METHODS

Sample Collection

Samples were collected from three forested sites in North Carolina: Raleigh (Piedmont), Bladen State Forest (Coastal Plain), and Fletcher (Blue Ridge Mountains). The Raleigh site consisted of three subsites, located 15-40 km apart: Schenck Forest, Lake Wheeler Field Lab, and Duke Forest. Termites were collected from wood debris in transect fashion from each site or subsite. Collection points, four to 11 per transect, were at least 15 m apart. In each site or subsite, there were two parallel transects, separated by 1 km. Ten to 50 workers and soldiers were collected per collection point. Samples were either frozen alive and held at –80°C or put in 100% ethanol and stored at 4°C until genetic analysis.

Molecular Genetic Methods

Genomic DNA was extracted from up to 20 individuals per colony from individual termites using either the Wizard Genome DNA Purification Kit (Promega) or the DNeasy Tissue Kit (Qiagen). Workers were genotyped at eight microsatellite loci according to the methods of Vargo (2000). MtDNA sequence analysis was performed using the methods of Jenkins et al. (1999b).

Colony Identification

The microsatellite genotypes of workers from nearby collection points along a transect were compared to determine the colony identity of all collection points. Workers from nearby collection points that had identical genotypes occurring at similar frequencies were grouped into a single colony.

Colony Classification

Based on microsatellite genotypes, colonies were classified into one of two main groups according to the following scheme:

Mendelian colonies were simple families, in which workers had Mendelian genotypes in expected ratios at all eight microsatellite loci.

Non Mendelian colonies were of three types: Mendelian genotypes in ratios deviating significantly from expected; non Mendelian genotypes consistent with being inbred families descended from simple families; colonies with multiple unrelated reproductives present.

Data Analysis

The average relatedness (*r*) for workers was estimated from the microsatellite genotype frequencies using the program RELATEDNESS (Queller and Goodnight, 1989). Total genetic variance was partitioned among the different levels of structure using the microsatellite data by calculating hierarchical *F*-statistics as implemented in the program Genetic Data Analysis (Lewis and Zaykin, 2000). The *F*-statistics and estimates of relatedness obtained for the different populations were compared to the values predicted by Thorne et al. (1999) and Bulmer et al. (2001) under several simulated breeding systems to identify the "best fit" of the potential breeding system in each transect.

RESULTS and DISCUSSION

As shown in Table 1, there were 92 collection points in all. Of these, in only two cases did colonies extend to a neighboring collection point, one colony in the Piedmont site and one in the Blue Ridge Mountains site. All other collection points were distinct colonies, indicating that colonies in these transects are fairly localized, rarely extending their foraging range beyond the 15 m interval between collection points. These results differ from those of Bulmer et al. (2001) on a population of *R. flavipes* studied in Massachusetts using allozymes and mtDNA markers. These authors found two of 22 colonies with foraging ranges extending over 50 m.

The proportion of Mendelian colonies ranged from 27% in the Coastal Plain site to 39% in the Piedmont site, with an overall proportion of 36% (Table 2). Of the 58 non Mendelian colonies, only one of the Blue Ridge Mountains colonies had more than four alleles at a locus, indicating the presence of multiple unrelated reproductives in this colony. All other non Mendelian colonies had genotypes consistent with being inbred colonies descended from Mendelian colonies. MtDNA sequence data were obtained for the Piedmont population only, and the data indicated that despite high variability of haplotypes within the population, all individuals within a colony had the same haplotype. Thus, the mtDNA data for the Piedmont site was consistent with

Table 1. Grouping of collection points into colonies

Site	No. collection points	No. colonies
Coastal Plain	22	22
Piedmont	57	56
Blue Ridge Mtns.	13	12
Total	92	90

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a single maternal origin for all colonies, both Mendelian and non-Mendelian, in this population. In their study, Bulmer et al. (2001) found that six (27%) of 22 colonies were Mendelian and three colonies (14%) had multiple mtDNA haplotypes indicating the presence of multiple unrelated females reproducing in the same colony. Jenkins et al. (1999a) reported finding multiple mtDNA haplotypes within a single group of foraging workers of an unspecified species of *Reticulitermes* in Georgia.

The values for the relatedness coefficient and the *F*-statistics for the Mendelian colonies are similar to the values expected for colonies comprised of simple families headed by a pair of outbred primary reproductives (Table 3). Only in the Blue Ridge Mountains site did F_{IT}, the coefficient of inbreeding for individuals relative to the total population, differ significantly from expected. These results suggest that in the Coastal Plain and Piedmont sites, Mendelian colonies are simple families headed by outbred primaries, whereas the high F_{IT} value for the Blue Ridge Mountains site suggests that primaries in these colonies may be somewhat related to each other, or come from inbred colonies. These results are similar to those reported by Bulmer et al. (2001) for Mendelian colonies in Massachusetts, which the authors concluded were simple families headed by outbred reproductives.

For the non-Mendelian colonies, the closest match between the empirically-derived values and the computer-generated values are those expected for colonies headed by a single pair of neotenic reproductive who are the direct progeny of the original primaries (Table 4). Again, the Blue Ridge Mountains site had the only values that differed significantly from the expected values, with significantly lower values of *r*, the coefficient of relatedness, and F_{CT}, the coefficient of

Table 2. Proportion of Mendelian and non-Mendelian colonies within populations

Site	Mendelian	non-Mendelian
Coastal Plain	27%	63%
Piedmont	39%	61%
Blue Ridge Mtns.	33%	67%
Total	36%	64%

Table 3. Comparison of empirically-derived values of the relatedness coefficient (*r*) and *F*-statistics for Mendelian colonies with values expected for simple families headed by a pair of outbred reproductives based on computer simulations (Thorne et al., 1999; Bulmer et al., 2001).

	r	F_{IT}	F_{CT}	F_{IC}
Empirical values				
Coastal Plain	0.54	0.05	0.26	-0.28
Piedmont	0.51	0.09	0.30	-0.30
Blue Ridge Mtns.	0.56	0.21*	0.34	-0.20
Expected values	0.50	0.00	0.25	-0.33

F_{IT} is the coefficient of inbreeding for individuals relative to the total population; F_{CT} is the coefficient of inbreeding for colonies relative to the total population; and F_{IC} is the coefficient of inbreeding for individuals relative to the colony. Empirical values that differed significantly from expected are indicated with an asterisk.

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Table 4. Comparison of empirically-derived values of the relatedness coefficient (r) and F-statistics for non-Mendelian colonies with values expected for inbred families headed by a single female neotenic reproductive and a single male neotenic reproductive who are the direct progeny of the original founding primaries reproductives.

	r	F_{IT}	F_{CT}	F_{IC}
Empirical results				
Coastal Plain	0.59	0.31	0.39	-0.13
Piedmont	0.54	0.19	0.34	-0.22
Blue Ridge Mtns.	0.42*	0.18	0.26*	-0.11
Expected values				
1 female, 1 male neotenic inbred for 1 generation	0.62	0.33	0.42	-0.14

The expected values are based on computer simulations by Thorne et al. (1999) and Bulmer et al. (2001). F_{IT} is the coefficient of inbreeding for individuals relative to the total population; F_{CT} is the coefficient of inbreeding for colonies relative to the total population; and F_{IC} is the coefficient of inbreeding for individuals relative to the colony. Empirical values that differed significantly from expected are indicated with an asterisk.

inbreeding of colonies relative to the total population. Nonetheless, the empirical values indicate that the number of reproductives in the non-Mendelian colonies is rather low and that they have not undergone more than a generation or two of inbreeding. The non-Mendelian colonies studied by Bulmer et al. (2001) showed much lower levels of r, as well as higher F_{IC} and F_{IT} values, all suggestive of higher numbers of reproductives inbred for more generations. These authors concluded that colonies had on the order of 10 or so neotenics that had undergone three or more generations of inbreeding.

In conclusion, colonies from all three undisturbed sites were fairly localized, with foraging ranges not extending beyond about 15 m in any one direction. Some one-third of the colonies were Mendelian, and estimates of the coefficient relatedness and F-statistics suggest that colonies in the Coastal Plain and Piedmont sites were headed by a single pair of outbred primaries, whereas those in the Blue Ridge Mountains site showed some signs of being slightly inbred. With only one exception, colonies were consistent with being inbred families descended from a single pair of primary reproductives. Based on estimates of the coefficient relatedness and F-statistics, these colonies contain very few neotenics that are not highly inbred. Current studies are underway to investigate the breeding structure of *R. flavipes* colonies in disturbed areas around buildings as well as the breeding system of other *Reticulitermes* spp.

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