SUBTERRANEAN TERMITE MOVEMENTS and RELATIONSHIPS OVER TIME: GENETIC CHARACTERIZATION of a FIELD SITE

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Abstract The purpose of this study was to examine the relatedness of *Reticulitermes flavipes* subterranean termites using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) DNA fingerprints and mitochondrial DNA (mtDNA) sequence from individual termites collected at four separate feeding sites on four different dates over six months. Data analyses demonstrated that, among all collections, gene flow was high (Nm>>1), DNA identity was > 94%, populations were skewed toward homozygotes (Fis > 0, Fit > 0) and there was a natural history of continued gene flow across all collections. Mitochondrial DNA sequence analysis resulted in three haplotypes or maternal lineages, M1, M2, and M3, which were disproportionately represented in the data set. We developed a model to explain our composite genetic data and discussed it and our findings in light of the hypothesis that *Reticulitermes flavipes* form polygnous colonies structured according to maternal kin-biased groups.

Key Words AFLP, SSR, mtDNA, COII gene sequence, gene flow

INTRODUCTION

DNA sequence data was first used in a longitudinal study of *Reticulitermes* subterranean termites in 1999 (Jenkins et al., 2001). The collection site, BH13, was on Sapelo Island, Georgia. The mitochondrial DNA (mtDNA) cytochrome oxidase II (COII) gene was sequenced from termites collected from this site and preserved in EtOH for up to six years. Comparison of the sequence data using both distance and parsimony analyses showed that termites collected from BH13 differed from month to month and year to year not only by maternal line, but also by species. One collection produced two maternal lineages. This data was unexpected for a monogyne colony and led to the hypothesis that *Reticulitermes* spp. could form polygnous colonies that are structured according to maternal kin-biased groups. The 1999 data set was limited to maternal sequence, which provided only a haploid picture of genetic relationships over time. It could not, therefore, provide a picture of diploid termite gene flow patterns over time.

The objective in this study was to provide a diploid and maternal, haploid picture of gene flow for *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) over time. Termite collections were made over a six-month period from four collection sites at a University of Georgia Research facility in Savannah, Georgia. Three types of DNA data were collected from each termite: amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) DNA fingerprints and mitochondrial DNA (mtDNA) cytochrome oxidase II (COII) gene sequence.

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MATERIALS and METHODS

Sample Collection

Reticulitermes flavipes were collected over a six-month period from four inspection ports located around a building identified as Shop on the Coastal Garden and Bamboo Farm Experiment Station, a University of Georgia facility in Savannah, Georgia. The sites were identified as Shop1, Shop 2, Shop 3, and Shop 6. There were 2 meters between Shop1 and Shop 2, 2 meters between Shop 2 and Shop 3, and approximately 8 meters between Shop 3 and Shop 6. Termites were collected from Shop1 on 08/18/99, 09/21/99, 11/15/99, from Shop 2 on 09/21/99, 11/15/99, 01/10/00, from Shop 3 on 09/21/99, 11/15/99, and from Shop 6 on 08/18/99, 11/15/99, and 01/10/00. All termites were preserved in 100% EtOH. Five termites per collection were randomly chosen for the study. Individual termites were analyzed for amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) DNA fingerprints and a partial mitochondrial DNA (mtDNA) cytochrome oxidase II (COII) gene (400 bp) sequence.

DNA Extraction

Individual termites, five per collection (55 total), were extracted using an E.Z.N.A7 Mollusc DNA Kit (Omega Bio-Tek, Inc.). DNA quality was checked using agarose gel electrophoresis. Samples were quantitated using a TKO 100 (Hoefer Scientific Instruments) spectrophotometer.

AFLP Analysis

Digestion/Ligation Reactions. An AFLP profile was generated for each sample according to a protocol adapted from Zhu et al. (1998) and Herbergs et al. (1999). DNA was digested with Taq1 and Pst1 at 37°C for one hour, then 65°C for 1 hour, followed by enzyme inactivation for 20 min at 80°C in a mixture containing 200 ng of DNA, 5U of Taq1 and 10U of Pst1 (New England Biolabs), 1X NEB3 Buffer (supplied with enzyme), 0.075 mg Bovine Serum Albumin in a total volume of 50 ul. A 10 ul ligation mix containing 11U of T4 DNA Ligase (New England Biolabs), 1X NEB3 Buffer, 6mM ATP and 5 pmol of each Pst1 adaptor (Table 1) and 50 pmol of each Taq1

Table 1. Nucleotide sequences for adaptors, pre-selective primers, and selective primers used in AFLP protocol using *Taq*1 and *Pst*1 restriction enzymes

Primer\Adaptor	Sequence
Taq1 adaptor 1	5'-GACGATGAGTCCTGAC-3'
Taq1 adaptor 2	3'-TACTCAGGACTGGC-5'
Pst1 adaptor 1	5'-CTCGTGACTGCGTACATGCA-3'
Pst1 adaptor 2	3'-CATCTGACGCATGT-5'
Taq1 Pre-selective primer	5'-GATGAGTCCTGACCGAA-3'
Pst1 Pre-selective primer	5'-AGACTGCGTACATGCAG-3'

	Extension	Label
Pst 1 Selective Primers		
P66	GAT	FAM
P67	GCA	TET
Taq1 Selective Primers		
Taq-ACT	ACT	–
Taq-AAG	AAG	–
Taq-ATA	ATA	–

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adaptor was then added to the sample and incubated at 16°C overnight. Before adding the adaptors to the ligation mixture, each pair of adaptors was mixed together, incubated at 95°C for 5 minutes, and cooled at room temperature for 10 minutes.

Pre-selective Reactions. Pre-selective reactions contained 1X PCR Buffer (supplied with Taq polymerase), 1.5 mM $MgCl_2$, 0.16mM dNTPs, 1U of Taq polymerase, 50 pmol of each pre-selective primer and 1.0 ul of digestion-ligation reaction in a total volume of 20 uL. The reactions were denatured at 95°C for 5 minutes followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s.

Selective Reactions. Selective reactions contained 1X PCR Buffer (supplied with Taq polymerase), 1.5 mM $MgCl_2$, 0.16 mM dNTPs, 1U of Taq polymerase, 50 ng of each selective primer and 0.5 ul of digestion-ligation reaction (diluted 1/10 with 0.1 mM Tris-EDTA) in a total volume of 20 ul. The reactions were denatured at 95°C for 5 minutes followed by a touchdown PCR of 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s with 0.7°C decrease in annealing temperature for an additional 20 cycles. This was followed by 24 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s. Selective primers were chosen based on degree of polymorphisms, reproducibility, and well defined peak patterns obtained from data generated in a screen of 35 primer pair combinations against a small subset of termite individuals. Selective reactions were electrophoresed using a Perkin Elmer 377 Sequencer according to manufacturer's protocol.

AFLP Data Analysis

AFLP data was processed using GeneScan7, Version 3.1.2 (Applied Biosystems). POPGENE Version 1.31 software was used to calculate the genetic identity for each section for each polymorphic loci and averaged across loci to get a within population estimate of genetic diversity and gene flow. Phylogenetic trees were constructed using the Neighbor Joining program with the UPGMA option in PHYLIP.

SSR Analysis

A SSR profile was generated for each sample according to a protocol adapted from Hopkins et al. (1999). Fluorescent labeled oligonucleotides were obtained commercially (Research Genetics) for forward primers of three primer pairs: RF6-1 (5'-FAM-AGACTTGGAGTGCACTGTTGTT) and RF21-1 (5'-HEX-CACACACGTTCGTTGTTTTG) (Vargo, 2000) and TE100B (5'-FAM-GCAGTGGTTGTACTAATGGCG-3'). Reverse primer for TE100B is 5'-CCACAAGTTCAGTCCCAGTCA-3' and for RF61 and RF211 are according to Vargo (2000). PCR reactions were accomplished in 25 ul volumes containing 1 X Promega PCR Buffer, 2.5 mM $MgCl_2$, 0.15 mM deoxynucleotide triphosphates, 0.03 units Taq DNA polymerase (Promega), 10 pmol of each primer and 25 ng of genomic DNA. Reactions were then denatured for 4 min. at 95°C, PCR continued for 25 cycles at 95°C for 1 min, 55°C for 2 min, 72°C for 2 min, and finally an extension at 72°C for 10 min. Electrophoresis on an ABI 377 automatic sequencer, detection of fluorescent products, and semi-automated fragment analysis were according to Hopkins et al. (1999).

SSR Data Analysis

SSR data was processed using GeneScan®. Microsat 1.5b (Eric Minch, Washington State University) software was used to calculate F-coefficients, determine gene flow, genetic identity and distance measure.

DNA Sequence Analysis

Sequence profiles of 401 bp of the COII gene were accomplished according to Jenkins et al. (2001). Sequence analyzed corresponded to bases 277 – 678 of *Reticulitermes flavipes* strain

RF53(4/95) COII gene in GenBank (gb # AF107489). Each unique sequence constituted a haplotype.

RESULTS

Gene Flow

The DNA fingerprints for each termite consisted of SSR and AFLP data sets. The F_{ST} estimate generated from the co-dominant SSR markers, which consisted of three primer pairs, was 0.0297 and the effective rate of gene flow or Nm >> 1 (8.17) was very high. These results indicated considerable gene flow between populations. F_{IS} and F_{IT} estimates from this data set were positive, 0.0176 and 0.0468 respectively. All three dominant AFLP markers (Table 1) were combined into one data set with 749 loci. Since there were 749 loci for the AFLP fingerprint, the coefficient of gene differentiation (G_{ST}) was generated. The G_{ST} = 0.2345 (Nei, 1987) and Nm >>1 (1. 633) estimates respectively were consistent with unrestricted gene flow among populations.

There was high genetic identity (Nei, 1972) among all individuals across all collections (Table 2). Collection 8/18/99 from Shop 1 and 9/21/99 from Shop 1 were the most alike (>99%) whereas collection 11/15/99 from Shop 2 and collection 9/21/99 from Shop 3 were the least alike genetically (>94%).

Phylogeny Analysis and Gene Flow

A phylogeny analysis of the AFLP data showed little sequence divergence among individuals (Figure1). The bootstrap values (100 pseudoreplicates) clearly demonstrated that individuals could not be separated. S1-1A99 and S1-2A99 appear to be significantly different from the other 53 individuals. This is the result of analysis with PHYLIP 3.6a. When these two individuals were taken out of the data set, another individual was randomly chosen by PHYLIP against which to compare all other individuals (tree not shown). A tree rooted with *Reticulitermes virginicus* (not shown) showed no significant separation among individuals. Thus, this unrooted tree (Figure 1) indicates free gene flow among all individuals in this study or a natural history of unimpeded gene flow.

Table 2. Nei's (1972) genetic identity above diagonal and genetic distance below diagonal. Numbers correspond to specific collection sites and dates of collection at those sites. These are: 1 = Shop1(8/18/99), 2 = Shop1 (9/21/99), 3 = Shop1 (11/15/99), 4 = Shop2 (9/21/99), 5 = Shop2 (11/15/99), 6 = Shop2 (01/10/00), 7 = Shop3 (9/21/99), 8 = Shop3 (11/15/99), 9 = Shop6 (8/18/99), 10 = Shop6 (11/15/99), 11 = Shop6 (01/10/00)

popID	1	2	3	4	5	6	7	8	9	10	11
1	****	0.9967	0.9968	0.9848	0.9801	0.9834	0.9517	0.9551	0.9677	0.9782	0.9798
2	0.0033	****	0.9958	0.9828	0.9782	0.9812	0.9492	0.9557	0.9685	0.9767	0.9784
3	0.0032	0.0042	****	0.9847	0.9806	0.9831	0.9504	0.9545	0.9659	0.9766	0.9805
4	0.0153	0.0173	0.0154	****	0.9883	0.9882	0.9461	0.9496	0.9617	0.9696	0.9730
5	0.0201	0.0220	0.0196	0.0118	****	0.9872	0.9422	0.9488	0.9580	0.9666	0.9708
6	0.0168	0.0190	0.0170	0.0119	0.0128	****	0.9467	0.9498	0.9604	0.9687	0.9709
7	0.0495	0.0521	0.0509	0.0554	0.0596	0.0548	****	0.9477	0.9488	0.9517	0.9528
8	0.0460	0.0453	0.0466	0.0517	0.0526	0.0515	0.0537	****	0.9660	0.9655	0.9673
9	0.0328	0.0320	0.0347	0.0390	0.0429	0.0404	0.0526	0.0345	****	0.9794	0.9725
10	0.0220	0.0235	0.0237	0.0308	0.0340	0.0318	0.0495	0.0351	0.0208	****	0.9831
11	0.0204	0.0218	0.0197	0.0274	0.0297	0.0296	0.0483	0.0332	0.0279	0.0171	****

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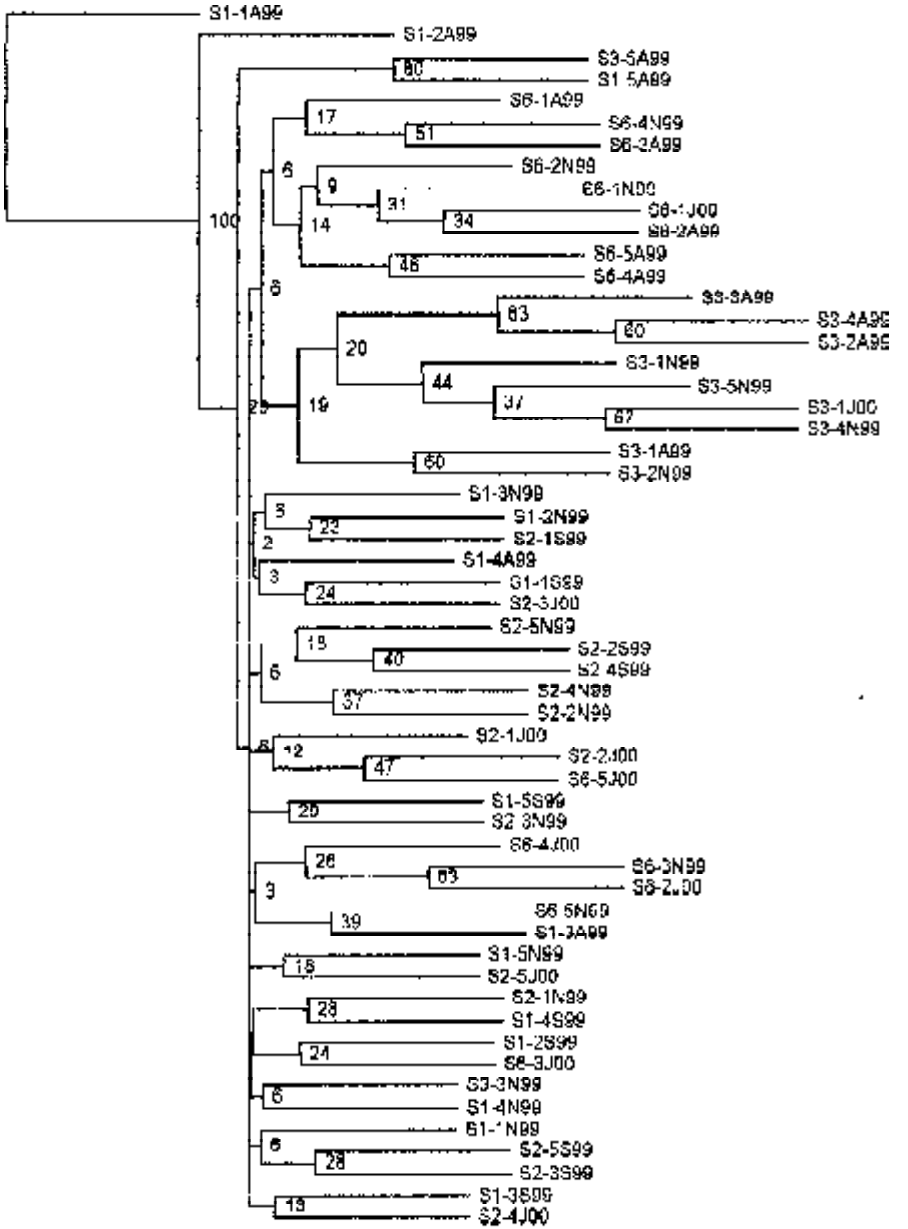

Figure 1. UPGMA unrooted tree of COII. Bootstrap values (100 pseudoreplicates) are indicated at the nodes. Individuals are represented by the site collected (S1 = Shop 1, S2 = Shop 2, S3 = Shop 3, and S6 = Shop 6), individual (1 , 2, 3 ,4, or 5), date collected (A99 = 8/128/99, S99 = 9/21/99, N99 = 11/15/99, and J00 = 01/10/00).

Mitochondrial DNA Haplotypes

Mitochondrial COII gene analysis resulted in three haplotypes: MI, M2, and M3. M1 was the dominant haplotype appearing in 6 of 8 collections: Shop 1 (8/18/99), Shop 1 (9/21/99), Shop 2 (9/21/99), Shop 2 (11/15/99), Shop 2 (1/10/00), Shop 6 (8/18/99). M2 appeared in 3 of 8 collections: Shop 3 (9/21/99), Shop6 (8/18/99), and Shop 6 (1/10/00). M3 appeared in 1 of 8 collections: Shop 3 (9/21/99). M2 and M3 were both found at site Shop 3 (9/21/99) and M1 and M2 were both found at site Shop 6 (8/18/99).

DISCUSSION

Gene Flow

Both co-dominant and dominant fingerprint data indicated the same trends. The difference in the SSR and AFLP values could be due to the difference in marker density, three loci vs. 749, co-dominant vs. dominant markers or selection on specific loci. The G_{ST} value for the AFLP data set did not change even when the SSR F_{IS} value was used in the analysis, which again could be due to the difference in marker density. Furthermore, SSR and AFLP DNA fingerprints were done on the same individual. Both AFLP and SSR fingerprints showed that populations were not in Hardy-Weinberg equilibrium. Thus, there was a skew toward homozygosity.

Across all collections we observed high gene flow and low genetic diversity, genetic identities > 94% (Table 2), a skew towards homozygotes ($F_{IS} > 0$, $F_{IT} > 0$) and a natural history commensurate with unimpeded gene flow (Fig. 1). These collections, therefore, could represent either a single colony or subpopulations of a single colony. At least three female lines, M1, M2, M3, are represented. The sampled population, therefore, consists of multiple breeding female lines that are genetically similar.

We consider these data representative of a meta-colony social structure in *Reticulitermes*. Since strict polygyny could result in reduced fecundity (Crozier and Pamilo, 1996), female lineages could have been introduced together or separately (Jenkins et al., 1999, 2001). This would result in an open (Clemént, 1986), meta-colony structure composed of multiple subpopulations of kin-biased maternal groups. Coalescence and/or pleometry in *Reticulitermes* spp. could also be the impetus, as previously discussed (Jenkins et al., 1999), for meta-colony formation. Maternally distinct groups could have come together in a cooperative manner, which has been demonstrated for other termite genera (Thorne, 1985). We offer a model (Figure 2), which demonstrates how our data could conform to a polygynous meta-colony structure organized around maternal kin-biased groups.

Model

The haplotype or maternal lineage M1 was most often recovered from collections. It was found in all but two collections, which would indicate that the area occupied by the progeny of this lineage is greater than the area occupied by the progeny of the other two lineages (Figure 2). This would suggest that the M1 lineage has either been part of the colony structure the longest or is the most fecund. M2 may have been introduced later into the colony structure or it could be an older maternal group in decline (Figure 2). M2 could also represent progeny of a less fecund line. Lineage M3 occupied the smallest subset of our collections. Thus the progeny of this lineage occupy only a small space and indeed, if not the result of sampling, could be vulnerable in terms of continued existence in the colony (Fig. 2). Since multiple lineages were found, M2 and M3 in collection Shop 3 (9/211/99) and M1 and M2 in collection Shop 6 (8/18/99), there appear to be some overlap of maternal groups, which suggest mtDNA introgression across maternal kin-biased boundaries and breeding groups.

Two caveats must be considered in this discussion. They include the size of the data set and the relatively closely spaced sites. We are presently studying widely disparate sites using the three

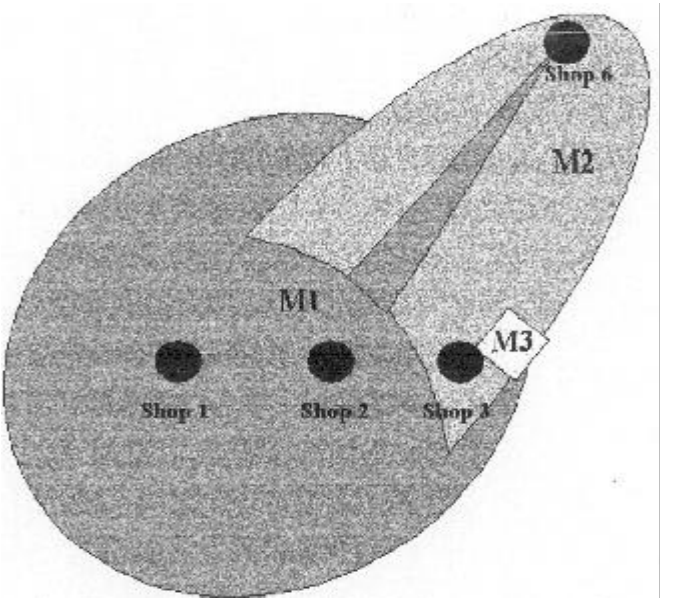

Figure 2. Three maternal lines were found across all collections: M1, M2, and M3. M1 was the most numerous. It was found in 7 of 9 collections at sites Shop 1, Shop 2, Shop 3, and Shop 4. M2 was found in 4 of 9 collections at sites Shop 1, Shop 2, and Shop 6. M3 was found in 1 of 9 collections at site Shop 2.

molecular markers for comparison with these data. Until we analyze these data and then compare them across multiple disparate sites, we are not clear whether gene flow within the genus *Reticulitermes* is unimpeded over their geographic range. To define if we have a single colony with multiple female lineages or multiple colonies of closely related individuals sharing a single food source, a multi-disciplinary research effort is required (Forschler and Jenkins,1999, 2000).

In conclusion, it was not until recently that DNA data was first used to suggest polygyny in *Reticulitermes* spp (Jenkins et al., 1999). This earlier data set of mtDNA COII sequences was limited in perspective to maternal lineages only. It and subsequent studies (Jenkins et al., 2001), however, resulted in a hypothesis that *Reticulitermes* spp form polygnous meta-colonies based on maternal kin-biased group. The AFLP and SSR data in this study did provide interesting results. They demonstrated high genetic identity and high gene flow that is indicative of a single colony. The mtDNA haplotypes superimposed on these results (Figure 2) could support the hypothesis of a polygnous colony structure for *Reticulitermes flavipes* consisting of maternal kin-biased groups (Figure 2). Yet, we also may have sampled multiple colonies, though closely related, which are simply sharing a single food source (Jenkins et al., 1999). We are continuing to sample disparate locations for comparison with these data using multiple DNA technologies.

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