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PRIMER NOTES

Polymorphism at trinucleotide microsatellite loci in the subterranean termite *Reticulitermes flavipes*

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The subterranean termite *Reticulitermes flavipes* (Rhinotermitidae) is widespread throughout the eastern U.S.A., where it is important both as a decomposer of wood and as an economic pest. The social organization and modes of colony foundation in *Reticulitermes* spp. are highly flexible (reviewed by Thorne 1998). However, the cryptic nesting and foraging habits of these subterranean species has made it difficult to conduct extensive studies of their social and spatial organization. Consequently, we have little information on the relative frequencies of the alternate forms of colony organization and modes of reproduction, or how these vary in response to ecological conditions.

Genetic markers have great potential for elucidating colony organization and population structure, but there have only been a few studies on *Reticulitermes* spp. using either allozymes (Clément 1981, 1984; Reilly 1987) or mitochondrial DNA sequence (Jenkins *et al*. 1998, 1999). To provide a sensitive tool for investigating colony and population structure, I developed microsatellite markers for *R. flavipes*.

Termites were collected from infested logs of pine and hardwood trees at various locations in North Carolina, USA. Heads of five workers from each of five colonies were used to construct the genomic library. DNA from pooled tissue was extracted using the Wizard Genomic DNA Purification Kit (Promega) after grinding in liquid nitrogen. Library construction and screening was performed largely following the protocol of Glenn (1996). The genomic DNA was digested with *Sau*3AI, and fragments 300–700 bp were selected for cloning into pZErO-2 plasmids (Invitrogen). I transformed TOP10 F′ cells to obtain a 20 000-clone library. Plated colonies were lifted onto nylon membranes which were probed with synthetic oligonucleotides consisting of 8–10 repeats of a trinucleotide motif rich in AT (AAT, AAC, ATC, AAG and ACT). Thirty-six positive clones were sequenced. Southern blots of plasmid DNA confirmed 36 positives, for which I obtained 23 sequences containing five or more repeats. From these sequences, 20 primer pairs were designed.

Microsatellite analysis was performed largely according to the methods of Oetting *et al*. (1995) in which the first 19 bp of the M13 forward sequencing primer (CACGACGTTGTAAAACGAC) is added to the 5′ end of one of the specific primers in each pair. The tail was attached to the left primer in each pair, except in the case of Rf 5–10, in which it was added to the right primer. A fluorescent labelled M13 (M13F-29/IRD 800, Li-Cor) primer was included in the polymerase chain reaction (PCR), yielding labelled product which was detected in a Li-Cor 4000 automated sequencer.

Heads of individual workers were pulverized in 0.5 mL reaction tubes with a plastic pestle. DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). The PCR reactions were set up in a 10-μL reaction mixture containing PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton®X-100), 2 mM $MgCl_2$, 200 μM each dNTP, 32 nM labelled M13F primer, 0.4 U *Taq* polymerase (Promega) and variable amounts of the specific primers (Table 1). All loci were amplified using a touchdown program on a PTC-100 (MJ Research, Watertown, MA, USA) thermal cycler: initial denaturation step at 94 °C (30 s), followed by 6 cycles at 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s), ramping down the annealing temperature 1 °C per cycle, and then 30 cycles at 94 °C (30 s), 54 °C (30 s) and 72 °C (30 s), with a final extension step at 72 °C (5 min). Following the addition of 5 μL formamide loading buffer to each sample, they were denatured at 90 °C for 3 min, snap cooled on ice and loaded onto polyacrylamide sequencing gels (8% Long Ranger, 7.5 M urea).

The population survey included colonies from two subpopulations in Wake County, North Carolina (Schenck Forest and Lake Wheeler Road Field Laboratory), and several colonies from various sites in North Carolina and northern South Carolina. One individual genotype per colony was used in the population survey. All nine microsatellites were polymorphic, with 2–23 alleles per locus (Table 2). The Schenck Forest samples came from an area of approximately 1.5 hectares, whereas the Lake Wheeler Road samples were collected from a 5-hectare area. Sampled colonies in each site were separated by at least 100 meters. All microsatellite loci were polymorphic in each site with between 2 and 12 alleles present, indicating a high level of genetic variability on a local scale.

Expected heterozygosity for the nine loci across the entire survey ranged from 0.30 to 0.93 (Table 2). Values in the two subpopulations were similar to the overall values. In most cases, observed heterozygosity levels were slightly lower than expected.

The primers of all nine microsatellite loci were also tested with a single individual from each of three colonies for two congeneric species: *R. virginicus* and *R. hageni*. Colonies of these two species were collected in Wake County, North Carolina. As shown in Table 3, all nine primer pairs yielded scorable PCR products in both species. Eight of the loci were polymorphic in *R. virginicus*, whereas six, all with eight or

Table 1 Primer sequences and characteristics of nine microsatellite loci

Locus	Core repeat*	Size* (bp)	No. of alleles	Frequency of most common allele	Concentration of primer (nM)	Primers (5′–3′)	GenBank accession number
Rf 1–3	$(ATT)_{10}(ATATT)_3$	217	9	0.38	200	F: TCCTAACTGTCTGGATGCGA	AF195930
					200	R: CAGAAGCCTTCCAACCAAAA	
Rf 3–1	$(ATA)_9$	111	9	0.35	100	F: CTTTTACGAACAAGACCCCG	AF195931
					100	R: CCCATATAGGGCTGTTGAGC	
Rf 5–10	$(CTT)_2AATGTCT(CTT)_{10}$	146	5	0.56	200	F: TTTAACAGTCAGGATATAGTAAGGCTG	AF195932
					200	R: GGAAACCTTCGGGATGAAAT	
Rf 6–1	$(GTT)_5(GAT)_8$	157	9	0.40	100	F: AGACTTGGAGTGCACTGTTGTT	AF195933
					200	R: GCCATCAGTCATCTCAGCAA	
Rf 11–1	$(TGA)_7TAA(TGA)_2$	234	6	0.50	100	F: TTCACCTTCAGGGATAAAGAACA	AF195934
					100	R: CTTATAAGGACAAGCGGGCA	
Rf 11–2	$TCATAA(TCA)_5$	270	3	0.77	100	F: GCTATTTATCCGAGCATGGC	AF195935
					100	R: CGGTCTTGTTCTGCACTTCA	
Rf 15–2	$(ATC)_6GTT(ATC)_3$	218	2	0.81	100	F: GCATTTCAATCGCATCCTTT	AF195936
					100	R: TGATGGTGATGGTGGTGATT	
Rf 21–1	$(CTA)_{21}$	225	19	0.20	20	F: CACACACGTTCGTTGTTTTG	AF195937
					20	R: CAAGAGGCGTGGGGTACTAA	
Rf 24–2	$(GTA)_{23}$	124	23	0.15	20	F: AGGATTAGCATTGACGGCAG	AF195938
					20	R: ATGCGCCTATAACCAGCAAC	

*Sequenced allele.

Table 2 Allele frequencies and heterozygosity levels for each population of *Reticulitermes flavipes* studied

Locus	Population	*N*	No. alleles	Size* (bp) of most common allele	Frequency of most common allele	H_O	H_E
Rf 1–3	Lake Wheeler	9	8	227	0.33	0.71	0.70
	Schenck Forest	8	6	227	0.44	0.62	0.74
	Others	7	4	227,221	0.36	0.71	0.70
	Total	24	9	227	0.38	0.67	0.77
Rf 3–1	Lake Wheeler	9	7	118	0.33	0.44	0.77
	Schenck Forest	8	5	124	0.37	0.62	0.73
	Others	7	4	124	0.43	0.57	0.69
	Total	24	9	124	0.35	0.54	0.75
Rf 5–10	Lake Wheeler	9	5	153	0.61	0.33	0.58
	Schenck Forest	8	5	153	0.50	0.87	0.67
	Others	7	3	153	0.57	0.43	0.58
	Total	24	5	153	0.56	0.54	0.62
Rf 6–1	Lake Wheeler	9	5	170	0.39	0.89	0.72
	Schenck Forest	8	7	170	0.44	0.62	0.73
	Others	7	6	170	0.43	0.71	0.71
	Total	24	9	170	0.40	0.75	0.73
Rf 11–1	Lake Wheeler	9	4	250	0.44	0.67	0.66
	Schenck Forest	8	5	250	0.63	0.75	0.56
	Others	7	5	250	0.43	0.71	0.97
	Total	24	6	250	0.50	0.71	0.67
Rf 11–2	Lake Wheeler	9	3	286	0.72	0.33	0.44
	Schenck Forest	8	2	286	0.88	0	0.22
	Others	7	3	286	0.71	0.29	0.45
	Total	24	3	286	0.77	0.21	0.38
Rf 15–2	Lake Wheeler	9	2	234	0.89	0.22	0.20
	Schenck Forest	8	2	234	0.88	0.25	0.22
	Others	7	2	234	0.64	0.43	0.46
	Total	24	2	234	0.81	0.29	0.30
Rf 21–1	Lake Wheeler	9	11	204,228	0.17	0.67	0.89
	Schenck Forest	8	9	204,219,264	0.19	0.62	0.86
	Others	7	9	207	0.21	0.71	0.87
	Total	24	19	204	0.17	0.67	0.92
Rf 24–2	Lake Wheeler	9	10	156	0.28	0.67	0.85
	Schenck Forest	8	12	195	0.19	1.0	0.9
	Others	7	11	129	0.21	0.72	0.89
	Total	24	23	129	0.15	0.79	0.93

*Size includes the 19-mer M13F sequence.
N, number of individuals sampled; H_O, observed heterozygosity; H_E, expected heterozygosity.

Table 3 Number of alleles at the microsatellite loci in *Reticulitermes virginicus* and *R. hageni*. Sample size was a single individual from each of three colonies

	Species	
Locus	*R. virginicus*	*R. hageni*
Rf 1–3	2	3
Rf 3–1	1	4
Rf 5–10	3	3
Rf 6–1	5	3
Rf 11–1	2	2
Rf 11–2	2	1
Rf 15–2	3	1
Rf 21–1	6	5
Rf 24–2	5	3

more perfect repeats, were polymorphic in *R. hageni*. Although the sample size used here was small, the results indicate that the primers successfully amplify scorable products in both species and that most loci are polymorphic.

The microsatellite markers developed in the present study are considerably more polymorphic than allozymes in *Reticulitermes* spp. Within a 1500 hectare site, Reilly (1987) found only 30% of 14 allozyme loci were polymorphic in *R. flavipes*, with only two alleles present at each polymorphic locus. Working on a much larger scale in Europe, Clément (1981) found a higher degree of polymorphism in *R. lucifugus* and *R. santonensis*; 52% of the loci examined were polymorphic, with between 2 and 8 alleles present at each variable locus. With a substantially higher degree of polymorphism, the present set of microsatellites offers a more powerful tool for fine-scale studies of colony-level and population genetic structure in *R. flavipes* and related species.

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Isolation and characterization of novel polymorphic tetra-nucleotide microsatellite markers from the blue marlin, *Makaira nigricans*

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Highly migratory istiophorid billfishes support valuable commercial and recreational fisheries throughout the world's tropical and subtropical marine waters. Understanding the underlying stock structure of billfishes is of great importance for conservation efforts. In blue marlin (*Makaira nigricans*) a number of polymorphic single-copy nuclear DNA markers were developed and utilized for an analysis of genetic stock structure (Buonaccorsi *et al*. 1999). Polymorphic nuclear microsatellite markers were desired to increase sensitivity of population comparisons.

Protocols for generating a microsatellite-enriched small fragment library of plasmid DNA clones followed Kijas *et al*. (1994) and Waldbieser (1995) with the following modifications and details. Total genomic DNA was isolated from frozen blue marlin heart tissue following protocols of Sambrook *et al*. (1989). Genomic DNA was digested with *Mbo*I (Gibco BRL) and fragments in the 500–1000 base pair (bp) size range were isolated by electrophoresis onto DE-81 paper (Whatman International Ltd). Priming sites were added to isolated DNA fragments by ligating genomic fragments to the plasmid vector Bluescript KS+ (Stratagene) using T4 DNA ligase (Stratagene). Asymmetric polymerase chain reaction (PCR) was performed to make the vector-insert construct single-stranded, following Kijas *et al*. (1994), for hybridization to a $(GATA)_5$ probe. Selected fragments were prepared for transformation following Kijas *et al*. (1994).

The resulting small fragment library was screened for microsatellite-containing clones using PCR (Gibco BRL) following protocols of Waldbieser (1995). Putatively recombinant clones served as template for amplification using a $(GATA)_5$ primer in combination with universal T7 forward and M13 reverse primers (Genosys). Since the $(GATA)_5$ primer annealed to many complementary sites at microsatellite-containing clones, smeared amplification products, corresponding to the length of the repeat region, were considered positive indicators for the presence of microsatellites. Positive clones were cycle-sequenced with Thermo Sequenase sequencing kits (Amersham) using infrared dye (IRD-800) labelled M13 universal primers (LiCor). Reaction products were electophoresed on an automated sequencer with fluorometric detection (LiCor).

Clones containing microsatellite motifs were selected for further analysis. Primers were designed from microsatellite flanking sequences and are summarized in Table 1. After initial amplification testing, either forward or reverse primers were labelled with fluorescent dye (IRD-800; LiCor) to allow for size detection of amplified products. PCR on test samples was performed using BRL PCR Reagent Systems (Gibco BRL), with a final concentration of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.025–0.030 μM each primer, approximately 5 ng template DNA, 0.0–2.5% formamide, and 0.025 units/μL *Taq* DNA polymerase in 10 μL total volume. Reactions were run in an MJ Research PTC-200 DNA Engine. Typical cycling conditions included an initial denaturation of 5 min at 95 °C, followed by 32 cycles of 1 min at annealing temperature (Table 1), 1 min at 72 °C, and 1 min at 95 °C. Final extension was carried out for 7 min at 72 °C. PCR products were electrophoresed on 25 cm, 8% LongRanger denaturing polyacrylamide gels (FMC BioProducts) and detected using a LiCor scanner. A sequence of known size was run in the centre and at each edge of the gel to