A Dominant and Undescribed Species of *Reticulitermes* in Sapelo Island (Georgia, USA)

by

David Sillam-Dussès[1,2] & Brian T. Forschler[1,*]

ABSTRACT

Based on mitochondrial DNA analysis, an undescribed species of *Reticulitermes*, morphologically close to *R. hageni*, was collected in Sapelo Island (Georgia, USA). This species is sympatric with *Reticulitermes flavipes* and *R. virginicus*. Aggressiveness has been observed between this new species towards *R. flavipes* and *R. virginicus*, which partly explained the predominance of this species in the studied area.

Keywords: Termites, Mitochondrial DNA, COII, Phylogeny

INTRODUCTION

Four species of *Reticulitermes* are known in the southeastern United States, including Georgia: *Reticulitermes flavipes* (Kollar), *R. virginicus* (Banks), *R. hageni* (Banks) and *R. malletei* (Nutting, 1990, Weesner 1965, 1970, Austin *et al*. 2007). The taxonomy of the genus is, however, a subject of continued investigation and revision (Nelson *et al*. 2008). Species identification in *Reticulitermes* is traditionally based on morphological characteristics of a seasonal adult form (alate) and a minority caste (soldier) but these stages are not always collected and ambiguities continue for anyone attempting to use dichotomous keys that use measurable morphometric characters that exhibit considerable overlap between species (Hostettler *et al*. 1995).

Cuticular hydrocarbon profiles have also been used for taxonomic differentiation within the *Reticulitermes* (Haverty *et al*. 1996, 1999, Haverty & Nelson, 1997, Page *et al*. 2002). Cuticular hydrocarbons are considered as valuable characters and have generally corroborated taxonomic designations

[1] Department of Entomology, University of Georgia, 413 Biological Sciences Building, Athens, GA 30602, USA

[2] Institute of Organic Chemistry and Biochemistry, Flemingovo nám. 2, 166 10 Praha, Czech Republic

*To whom correspondence should be sent; bfor@uga.edu

based on genetic markers (Jenkins *et al*. 2000, Kutnik *et al*. 2004, Copren *et al*. 2005). They should, however, be evaluated carefully because profiles may be generated by environmental conditions (Woodrow *et al*. 2000) or by the cohabitation of different species (Vauchot *et al*. 1995, 1998).

Genetic markers are useful in difficult taxonomic groups and Cytochrome Oxidase II (COII) of the mitochondrial DNA has been successfully used to identify *Reticulitermes* species (Austin *et al*. 2002, Jenkins *et al*. 1999, 2001, Lo *et al*. 2000, Miura *et al*. 1998, 2000, Su *et al*. 2006, Vargo & Carlson 2006). Mitochondrial DNA is not only useful for species identification, but it may also be used to describe the spatial and social organization in *Reticulitermes* (Jenkins *et al*. 1999, Forschler & Jenkins 1999). It is generally assumed that *Reticulitermes* populations are established by a single queen. Thus, each population should have a unique mitochondrial DNA genotype. Therefore, each colony carries the unique mitochondrial DNA of the foundress, and we can apply mitochondrial DNA to differentiate populations of termites.

We used mitochondrial DNA to conduct an extensive population genetic study in part to distinguish between *Reticulitermes* species and to assess the distribution of the sympatric subterranean termite populations of this genus across a wildland area on Sapelo Island (Georgia).

MATERIALS AND METHODS

The study involved 4 test plots located within 6 m of each other the north east side of Sapelo Island, Georgia (N 31°23', O 81°16'). Three different kinds of termite food resources were used and these included; termite D-tecktor – 5 pads of paper (12 x 8 x 0.1 cm) in a plastic sleeve with a hole cut in the sleeve to allow ground contact, inspection ports (as described in Forschler and Townsend (1996), and large food resources - 4 pieces of dimensional lumber (90 x 13 x 4 cm). Termite D-tecktors and the large food resources were placed on the soil surface while the inspection ports were buried approximately 17-cm below the soil surface. The number of stations at each plot was 172 termite D-tecktors, 15 inspection ports and 4 large food resources (Fig. 1). Installation of the plots was conducted in September 2004 with termite collections commencing the following month and were conducted monthly, ending in July 2006 (no collections were made in February 2004, January 2006, May 2006, and June 2006). The large food resources were an exception in that they

were involved in sampling for 18 months, ending in April 2006. Termites, when present during the monthly inspections, were placed in 70% ethanol and returned to the laboratory for DNA extraction.

DNA was extracted from workers using the E.Z.N.A. Mollusc DNA kit (Omega Bio-Tek, Inc., Doraville, GA). One worker was extracted from the collections made at each location on every date. Polymerase chain reaction (PCR) was used to amplify about 800 bp fragment of the COII gene from each termite. Oligonucleotide primers TL2-J-3037 (alias A-t Leu) (5'-ATG-GCAGATTAGTGCAATGG-3') and TK-N-3785 (alias B-t Lys) (5'-GTT-

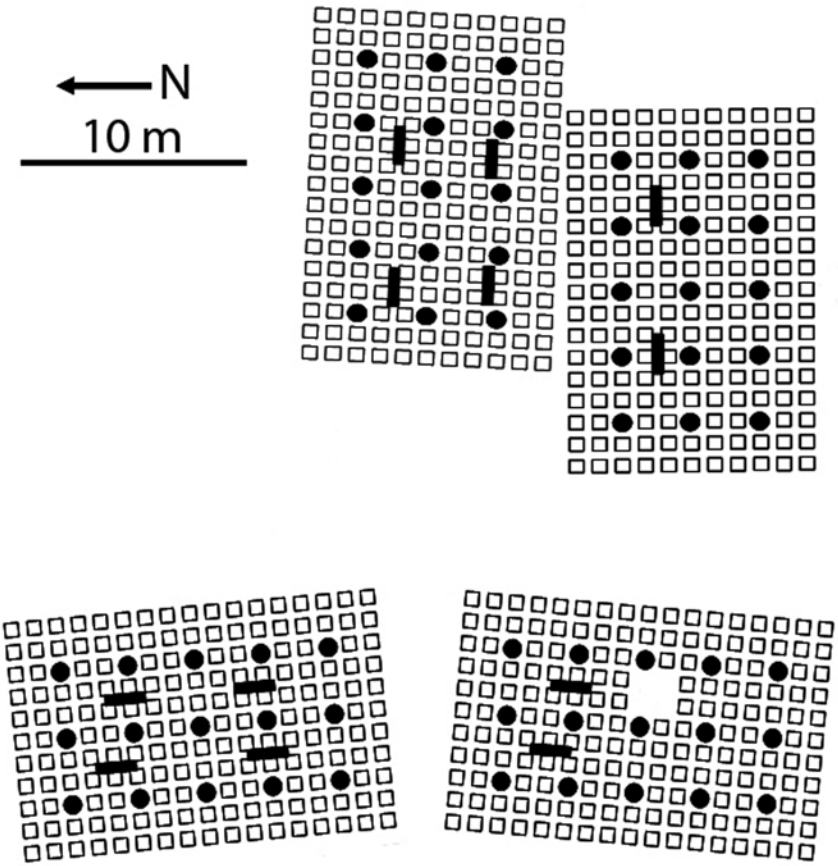

Fig. 1: Diagram of the 4 plots involved in this study. Squares are termite D-tecktors (688 in total, 5 pads of paper 12 x 8 x 0.1 cm in a plastic sleeve with a hole), circles are inspection ports (60 in total, 3 pieces of pine wood 12 x 4 x 3 cm), rectangles are large food resources (16 in total, 3 logs of pine 90 x 13 x 4 cm). Termite D-tecktors are 1 m apart.

TAAGAGACCAGTACTTG-3') (Liu & Beckenbach 1992) were used to amplify as well as to prime the sequencing reactions. PCR was performed in a standard 25 µl reaction. Amplification was accomplished in a T3 Thermocyler Biometra. It included a precycle denaturation at 94°C for 1 min, a postcycle extension at 72°C for 5 min, and 30 cycles of a standard three-step PCR. Each cycle consisted of 94°C for 1 min, 51°C annealing for 1 min, and 72°C extension for 2 min. Fragments were treated with exonuclease I (10 U/µl) and shrimp alkaline phosphatase (1 U/µl) and were incubated at 37°C for 15 min, then at 80°C for 15 min. All PCR products were further purified according to protocol with the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). Samples of the amplified DNA fragment from individual termites were sent to the Sequencing and Synthesis Facility (SSF) at McLab (San Francisco, CA) for sequencing in both directions. Individual electropherograms were edited and contigs formed using Sequencer 4.1.4 software (Gene Codes Corp., Ann Arbor, MI). Sequences that were exactly alike were compiled into a consensus sequence prior to alignment. CLUSTAL W (http://cbi.labri.fr) (Thompson *et al*., 1994) was used to align 716 COII nucleotides. Phylogenetic analysis was done using algorithms of PHYLIP (Phylogeny Inference Package) (Felsenstein 1989) to generate rooted maximum parsimony and neighbor-joining analysis (Saitou & Nei 1987). Node support was estimated by searching on 1,000 non parametric bootstrap replicates (Hillis *et al*. 1996). Bootstrap node support >70% was considered strong (Hillis & Bull, 1993). Maximum likelihood analysis was also conducted using PAUP* 4.0b10 (Swofford 2001). Gaps were treated as missing data. The default likelihood parameter settings were used (HKY85 six-parameter model of nucleotide substitution, empirical base frequencies, and transition/transversion ratio set to 2:1) to carry out a heuristic search. Bootstrap values were based on 100 replicates. We also employed Bayesian analysis (Ronquist & Huelsenbeck 2003) to estimate tree topology and posterior probabilities for each node and for nodes recovered in a majority of the trees. Trees were generated with Treeview (Page, 1996). All sequences were analysed using *Coptotermes formosanus* (GenBank accession number AY683221) as an outgroup. Sequences of the 4 species that are sympatric throughout Georgia, *Reticulitermes hageni*, *R. virginicus, R. flavipes*, and *R. malletei*, were added in all the three analysis and identified to species using

soldier morphology (n=1920 samples) and/or alignment with known mitochondrial DNA congruence based on previous studies (Scheffrahn & Su 1994, Hostettler *et al*. 1995, Forschler & Jenkins 1999, Jenkins *et al*. 2000). The Genbank accession numbers are EU689026, EU689027, AF107489, GU550074, respectively.

RESULTS

A total of 2640 *Reticulitermes* COII sequences were analyzed that provided 23 maternal lineages (ML's), labeled A to W. There were 107 polymorphic sites from the 23 ML's and the number of base pair (bp) differences ranged from 1 to 54 bp's (Fig. 2). Identification of species based on soldier morphology indicated that three species were collected from the various plots - *Reticulitermes flavipes*, *R. virginicus* and *R. hageni* (Scheffrahn & Su 1994). One and only one species identification based on soldier characters was obtained for each ML. Interestingly, the ML's grouped by neighbor-joining, maximum parsimony, maximum likelihood and Bayesian analysis clustered identically, with strong bootstrap values, indicative of species groups when reference sequences are included (Fig. 3). Phylogenetic analyses correlate with morphological determination, except for the last subclade that indicates an unidentified species (*R. hageni* according to soldier morphology). Ten ML's belonged to the clade

Species	COII	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
R. sp.	A		1	47	54	15	21	52	46	50	19	50	19	18	18	17	45	50	39	16	53	20	47	39
R. sp.	B			46	53	14	20	51	45	49	18	49	18	17	17	16	44	49	38	16	52	19	47	38
R. flavipes	C				23	43	46	21	7	17	46	15	45	45	45	46	6	16	46	46	19	47	20	48
R. flavipes	D					48	41	8	25	24	49	22	50	50	50	51	25	23	45	51	23	53	26	47
R. sp.	E						8	48	22	44	6	46	4	5	5	4	41	46	31	5	47	8	49	31
R. sp.	F							51	45	49	6	48	4	3	5	4	46	49	33	5	50	7	47	34
R. flavipes	G								23	22	51	20	50	50	50	51	23	21	47	50	23	52	24	49
R. flavipes	H									20	47	20	44	44	44	45	3	20	45	44	22	50	23	48
R. flavipes	I										47	8	46	46	46	46	19	8	47	46	7	49	16	48
R. sp.	J											48	4	3	3	2	46	49	34	3	50	1	47	34
R. flavipes	K												47	46	47	48	19	3	48	47	9	49	14	49
R. sp.	L													1	3	2	43	48	31	3	49	5	46	34
R. sp.	M														2	1	44	49	31	3	49	4	46	33
R. sp.	N															1	43	48	33	2	49	4	46	33
R. sp.	O																44	49	31	1	50	3	48	33
R. flavipes	P																	20	44	52	22	47	23	46
R. flavipes	Q																		47	48	9	51	16	52
R. virginicus	R																			34	48	36	46	4
R. sp.	S																				16	4	46	35
R. flavipes	T																					20	18	50
R. sp.	U																						48	35
R. flavipes	V																							47
R. virginicus	W																							

Fig. 2: Number of different bases between all the ML's of the study.

of *R. flavipes* while 11 belonged to the clade of *Reticulitermes* sp., and 2 ML's belonged to the clade of *R. virginicus* (Fig. 3).

The ML's identified as belonging to the *Reticulitermes* sp. clade occupied more stations (the range was 65-80% of stations occupied by this species every month, Mean = 73 ML's/month) than *R. flavipes* (range = 18-34.8%, Mean = 26.8) or *R. virginicus* (range = 0-2.2%, Mean = 0.3) (Table 1). *R. flavipes* and *Reticulitermes* sp. could be found in all the plots, whereas *R. virginicus* was collected only from two plots.

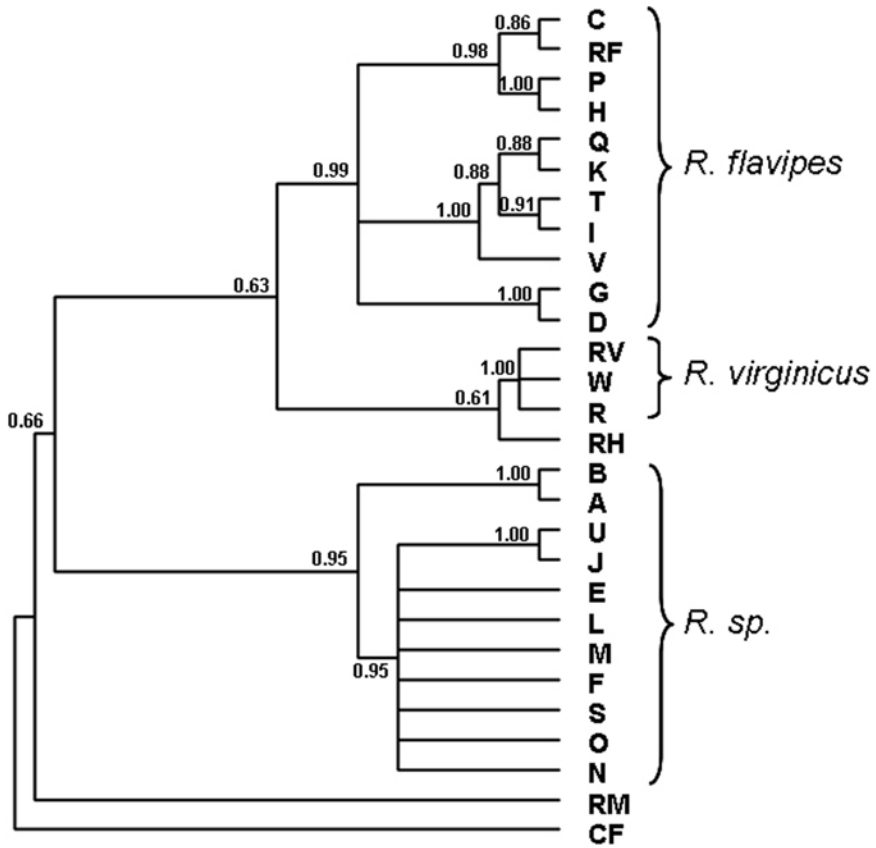

Fig. 3: Bayesian Topology of Bayesian majority rules consensus tree of 100,000 trees showing the genetic relationships among the maternal lineages (from A to W) (RH, *Reticulitermes hageni*; RF, *Reticulitermes flavipes*; RV, *Reticulitermes virginicus,* RM, *Reticulitermes malletei*). Numbers on the branches indicate posterior probabilities for key nodes. *Coptotermes formosanus* (CF) is the outgroup. Neighbor-joining, maximum parsimony, maximum likelihood analysis (not shown) clustered identically.

Table 1: Distribution of the species (R. sp., undescribed species; RF, *Reticulitermes flavipes*; RV, *Reticulitermes virginicus*) on all stations over the entire study (from September 2004 to July 2006). The table indicates the number of stations in which termites were collected.

		2004				2005											2006					
Species	ML	Sep	Oct	Nov	Dec	Jan	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Feb	Mar	Apr	Jul	Total	Average
R. sp.	A	13	12	15	13	17	18	17	22	27	32	26	29	14	17	3	12	12	12	22	333	
	B	10	19	24	11	11	13	25	23	39	35	38	51	26	26	21	26	30	12	40	480	
	E	9	9	9	4	5	18	18	16	18	24	17	16	12	13	4	6	9	9	12	228	
	F	2	3	5	1	1	5	2	3	2	5	4	11	3	3	1	3	4	2	4	64	
	J	11	7	14	8	7	16	12	17	12	20	19	24	12	11	8	11	12	6	17	244	
	L	5	7	4	0	1	6	3	1	6	5	6	8	3	2	2	0	2	2	9	72	
	M	6	4	8	7	7	13	14	10	16	11	9	16	8	11	7	7	6	4	15	179	
	N	13	6	13	12	13	14	19	20	26	28	18	37	11	15	5	13	9	6	16	294	
	O	0	0	0	2	1	0	2	0	0	2	0	2	2	1	2	1	0	1	1	17	
	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	
	U	0	0	0	0	0	0	0	0	0	2	0	1	1	2	1	1	1	1	0	10	
Total		69	67	92	58	63	103	112	112	146	164	137	195	92	101	54	80	85	55	137	1922	
% Total		79	71	71	65	67	80	70	69	70	72	70	73	77	70	71	71	79	77	75		73
RF	C	4	10	20	18	19	4	24	34	34	33	40	35	15	16	10	15	9	7	13	360	
	D	7	7	7	4	0	5	5	7	8	9	7	19	8	15	8	10	8	6	15	155	
	G	5	6	4	4	2	4	5	3	6	9	8	10	3	9	4	6	4	1	5	98	
	H	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	3	
	I	1	3	5	4	7	9	8	3	7	9	0	4	0	1	0	1	1	2	5	70	
	K	1	1	1	0	0	2	2	0	1	2	0	1	0	1	0	0	0	0	1	13	
	P	0	0	0	1	1	0	0	1	2	0	0	0	1	1	0	0	0	0	0	7	
	Q	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
	T	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	
	V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	
Total		18	27	37	31	30	24	45	49	59	63	55	69	27	43	22	32	22	16	40	709	
% Total		20	28	28	34	32	18	28	30	28	27	28	26	22	29	28	28	20	22	22		27
RV	R	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	3	8	
	W	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	
Total		0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	4	9	
% Total		0	0	0	0	0	0.8	0.6	0.6	0.5	0	0.5	0	0	0	0	0	0	0	2.2		0.3

DISCUSSION

In this study, we collected three *Reticulitermes* species: *Reticulitermes flavipes*, *R. virginicus*, and what we believe to be an undescribed species. This new species is morphologically close to *R. hageni* but it is clearly different from all the species already described from Southeastern United States based on mitochondrial DNA. Previous collections from Sapelo Island were described using cuticular hydrocarbon phenotypes, mitochondrial DNA phylogenetic analyses, in addition to alate and soldier morphology (Haverty *et al*. 1996, Jenkins *et al*. 2000). From those studies two specimens, BH25 and HH11, were claimed to represent at least one new taxon in *Reticulitermes* (Haverty *et al*. 1996, Jenkins *et al*. 2000). Based on maximum parsimony analysis, the new species collected may belong to the same taxon than BH25, but probably not to the taxon of HH11 (results not shown).

We collected two of the four species described from the SE US during this study - *R. flavipes* and *R. virginicus* - whereas *R. hageni* and *R. malletei* were not collected. Although a low level of aggression between *Reticulitermes* colonies has been reported in the literature (Bulmer & Traniello 2002, Clément 1986, Grace 1996, Polizzi & Forschler 1998), we observed strong displays (100% of pairing) of aggression between *Reticulitermes* sp. towards *R. flavipes* and *R. virginicus* (results not shown). This behavior consolidates the hypothesis of our *R. species* being an undescribed but valid species.

In our study, we observed a high variety of ML's on a relatively small spatial scale. Mitochondrial DNA is polymorphic enough to reveal population differentiation on a small scale, and is also a successful marker to discriminate between species. This powerful tool is particularly useful when morphological data are not available. Alates are occasionally collected and our study clearly showed that soldier morphology is not reliable, in part, because there are no keys that include *R. malletei*.

Understanding the population structure of subterranean termites has become essential for pest management, especially in the genus *Reticulitermes* which is the most economically important group of wood-destroying insects in the United States (Su & Scheffrahn 1990). The occurrence of an undescribed species which is predominant in the study area is important for development of novel pest management strategies and further ecological research.

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