

Mitochondrial Gene Sequence Questions
***Reticulitermes* sp. Social Structure**
(Isoptera: Rhinotermitidae)

by

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ABSTRACT

Termites are unique among the social insects. Isoptera is the only Order within the Insecta comprised entirely of diploid animals whose polyethic, caste-filled societies contain both male and female workers. The cryptic lifestyle, complex caste system, dynamic developmental pathways, and social composition present significant challenges to scientists attempting to delineate the development of and interactions within subterranean termite societies. For example, it is assumed that subterranean termite populations from the economically important genus *Reticulitermes* are closed and established by a single queen and a single king. As the colony grows progeny of the royal progenitors, secondary reproductives, develop that mate with their nest-mates to produce progeny (1, p. 138). This view of subterranean termite social structure has been the foundation for discussion and research on topics as diverse as eusocial evolution and control tactics based on population management. Although secondary reproductives may be numerous in *Reticulitermes* populations, female secondary reproductives are direct descendants of the queen and, as such, both inherit and pass on her mtDNA legacy. Thus each colony should present the mtDNA fingerprint of the founding queen. Here we present maternally inherited mitochondrial DNA (mtDNA) sequence from randomly chosen individual termites collected over three years from a single inspection port (feeding site). Phylogenetic analyses of cytochrome oxidase II (COII)

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sequence demonstrated unique mtDNA genotypes among dates and within a specific date. These unexpected results are the first mtDNA data to challenge the accepted assumption of a closed *Reticulitermes* colony with a single matriarch.

INTRODUCTION

Long-term field studies of subterranean termite biology generally rely on inspection ports or monitoring stations (purposefully placed feeding sites) maintained for the collection of termites at intervals over time (Grace *et al.* 1989; Su *et al.* 1993; Forschler 1996; Forschler and Townsend 1996). These inspection ports have been utilized to determine subterranean termite wood consumption rates, as well as, colony related parameters including population size estimates, foraging distances, and related use of feeding sites (Grace *et al.* 1989; Su *et al.* 1993; Forschler 1996). Despite the fact that a reliable definition of a colony is critical to understanding subterranean termite biology, colony associations continue to be based on field protocols that use empirical data and assume a single matriarch per colony. Traditional protocols establish subterranean termite colony associations using one or more of the following techniques: morphometric comparisons, behavioral bioassays, and mark-release-recapture (MRR) experiments. However, all three of the aforementioned techniques have been reported to provide ambiguous information (Haverty *et al.* 1996; Thorne *et al.* 1996; Polizzi and Forschler 1998; Polizzi and Forschler in press). The taxonomy of the genus *Reticulitermes* based on classical morphometric characters is not well defined and will likely be revised to include new species descriptions (Haverty *et al.* 1996; Thorne 1998). Defining colony relationships based on kin recognition using behavioral assays that record agonistic encounters also provide equivocal results (Polizzi and Forschler 1998; in press). MRR techniques have limitations due to lack-of-fit between the known biology of the system and the model assumptions used to estimate population size or colony composition (Thorpe *et al.* 1996). Despite these limitations, however, subterranean termite colonies have been defined, in the scientific literature, as those termites known, only from MRR studies, to use the same group of inspection ports (Su *et al.* 1993; Forschler 1996; Forschler and Townsend 1996). This pragmatic definition of a colony uses the appearance of one marked individual in a separate, non-release-site inspection port as verification of the related use of feeding sites by a single subterranean termite colony. The MRR-derived concept of a subterranean termite colony has even formed the basis for evaluation of *Reticulitermes* population management control strategies (Su and Scheffrahn 1998).

Since 1993 we have studied subterranean termite biology using established inspection ports that were monitored on a monthly basis from field sites in four soil provinces in Georgia, USA. We have collected termites from a single inspection port located in the Atlantic Coastal Flatwoods soil province on Sapelo Island, Georgia, designated BH13, since August, 1994. In February, 1996 the mean live weight of the termites collected from BH13 increased by 30%; and, examination of soldier morphology indicated that a species change had occurred - from *Reticulitermes virginicus* (1994-95) to *R. flavipes*. Examination of mean live weight and soldier morphology indicated that there was another species change in August 1998 from *R. flavipes* to *R. hageni*. Repeated attempts to link termites collected from BH13 to other inspection ports using MRR failed to indicate any connections after 1995. Therefore, using conventional nonmolecular techniques, we assumed that BH13 was occupied, in succession, by single *Reticulitermes virginicus*, *R. flavipes* and *R. hageni* colonies from 1994 to 1998.

The traditional, nonmolecular BH13 data exemplifies our experience with numerous inspection ports ($N > 100$) we have maintained at our other field sites - that subterranean termite selection of, and movement between, feeding areas is dynamic and changeable - and highlighted the fact that DNA could effectively be used to address the question of subterranean colony structure. As Avise (1994, p. 15) points out, "molecular markers are used most intelligently when they address controversial areas or when they are employed to analyze problems in natural history and evolution that have proven beyond the purview of traditional nonmolecular observation." A previous mtDNA study of *Reticulitermes* spp. from several inspection ports prompted a reevaluation of our BH13 field data and provided the impetus for this study (Jenkins *et al.* 1999). We decided to examine the mitochondrial COII gene sequence from individual termites collected over time from the BH13 inspection port. Each of the COII sequences are identified here as a combination of the inspection port and collection date, i.e. BH13(11/94). Our objective was to examine the maternal relatedness among/between termites collected over time from a single feeding site (inspection port) and was based on two concepts. First, the relatedness between and among individuals of a monogyne colony collected from an inspection port over time could be examined, and/or verified, using maternally inherited mitochondrial DNA (mtDNA). Second, the COII gene in offspring of a single female progenitor should have little or no DNA sequence differentiation over the three years of the study.

METHODS

Amplification and Sequencing

Individual termite DNA was amplified and sequenced as follows: three from 11/94, five from 4/96, five from 5/96, three from 6/96, three from 10/96, and four from 8/98. DNA templates for PCR amplification consisted of total nucleic acids isolated from individual whole worker termites (Jenkins *et al.* 1999) that were live or preserved in alcohol. DNA extraction was accomplished as described (Liu and Beckenbach 1992; Jenkins *et al.* 1999). Oligonucleotide primers A-tLEU and B-tLYS (Liu and Beckenbach 1992) were used to amplify as well as to prime the sequencing reactions for both strands of a 685-bp fragment of the COII gene. A BLAST search (Altschul 1990) confirmed insect mtDNA sequence (Jenkins *et al.* 1999). Three single date inspection port collections from other sites were included as possible species markers. Andy1(8/97), N4(4/97), and RF53(4/95) are considered, respectively, *R. hageni*, *R. virginicus*, and *R. flavipes*, as determined by both elate and soldier morphology using individuals recovered from a single inspection port on a specific date. *Coptotermes formosanus* sequence, designated CF(8/97), was used as the outgroup. All consensus sequences have been deposited in GenBank (Accession #s AF107479, AF107480-AF107489).

PCR amplification was performed in 50- μ l reactions with a minimum of 10 ng of total genomic DNA, 1 pmol of the two primers, 2.0 mM MgCl₂, 1.6 mM dNTPs and 0.05 U/ \sim 1 *Taq* DNA polymerase. Amplification was accomplished in a Perkin-Elmer Gene Amp PCR system 9600 (PE applied Biosystems, Foster City, Calif.), and included a precycle denaturation at 94°C for 2 min., a postcycle extension at 70°C for 7 min., and 25 cycles of a standard 3-step PCR conditions (50°C annealing). Fragments were treated with exonuclease I (10 U/ \sim 1) and shrimp alkaline phosphatase (1 U/ \sim 1) and incubated in a Perkin-Elmer Gene Amp PCR system 9600 first at 37°C for 15 min., then at 80°C for 15 min. in order to remove primers and inactivate dNTPs left over from the PCR reaction respectively. DNA (10-20 ng/ 100 bp PCR Product) was prepared for sequencing on the ABI 373 automated DNA sequencer (PE Applied Biosystems, Foster City, Calif.) where reactions were fractionated and initial base assignments made by the ABI automated system.

Statistical Analysis

Sequencer individual chromatograms were edited; contigs made and alignments done; consensus sequences assigned; multiple consensus sequences of all samples were aligned with Malign (Hein 1989); aligned

sequences were reformatted for PHYLIP (Felsenstein 1993) and bootstrapped 1000 times (Felsenstein 1985). DNADIST (Kimura 2-parameter model), NEMHBOR to infer trees and CONSENSE for a consensus tree produced a rooted Neighbor Joining (NJ) phylogram (Felsenstein 1993). The same bootstrapped data was then used with DNAPARS and CONSENSE to produce the Parsimony tree (P). The unbootstrapped data were run through DNAML (Felsenstein 1993) to get a Maximum Likelihood (ML) tree. All figures were drawn with TREEVIEW PPC (Page 1996).

RESULTS AND DISCUSSION

The COII gene in all three individuals sequenced from BH13(11/94) were identical. Three of the five individuals from BH13(4/96), designated BH13(4/96)-a, had identically unique sequence and two of the five individuals, BH13(4/96)-b, had identically unique sequence (Figs. 1, 2, 3). The COII gene in all individuals sampled from BH13(5/96), (6/96), (10/96), and (8/98) were identical within sample date and different between dates (Figs. 1, 2, 3). Additional sequence data were included in the analysis as species markers, and are identified as Andyl(8/97), N4(4/97), and RF53(4/95). These sequences were obtained from 2-5 individual termites that were identified to species using both alate (adult) and soldier morphology and represent *R. hageni*, *R. virginicus* and *R. flavipes* respectively. *Coptotermes formosanus*, CF(8/97), was the outgroup against which all *Reticulitermes* specimens were compared. All species and outgroup sequences were identical within samples but different between samples (Figs. 1, 2, 3).

Both phonetic and cladistic analyses of the mitochondrial sequence data show bootstrap support for different mtDNA genotypes from the 11/94 and 8/98 collections (Figs. 1, 2). All three trees grouped these two collections into separate clades. BH13(8/98) grouped with *R. hageni* (Andyl(8/97)) as expected. Highlighting the taxonomic uncertainty, BH13(11/94), identified with conventional techniques as *R. virginicus*, did not group, as expected, with N4(4/97). There is bootstrap support, however, for it being different from all other BH13 collections (Figs. 1, 2).

Unexpectedly during 1996, when we had assumed a single species and one monogyne colony, there were five mtDNA genotypes found (Figs. 1, 2, 3). All three trees group collections in 4/96-b, 5/96, 6/96 and 10/96 similarly, but with varying support. NJ provided good bootstrap support (Fig. 1) and P bootstrap support was moderate to poor (Fig. 2), which could be an anomaly of both sample size and methodology. The BH13(4/96) collection demonstrates different mitochondrial

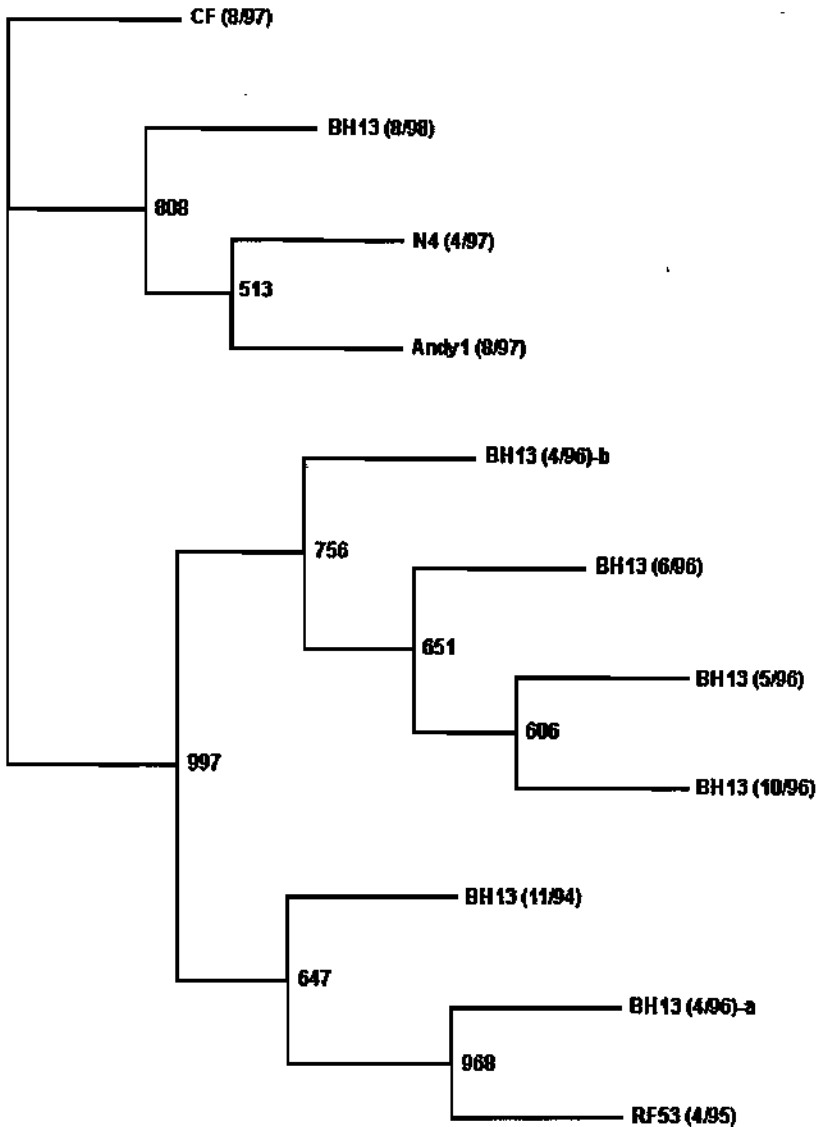


Fig. 1. Rooted Neighbor joining phylogram of the relationships of the CO1:1 nucleotide sequences among collections of *Reticulitermes* spp. made over time from the BH13 inspection port on Sapelo Island, Georgia U.S.A. was constructed using PHYLIP programs (20): The phylogram was bootstrapped 1000 times, then DNADIST with the Kimura 2-parameter model, plus NEIGHBOR with CONSENSE for the consensus tree and the bootstrap p-values (20). The phylogram was constructed with TREEVIEW PPC (21).

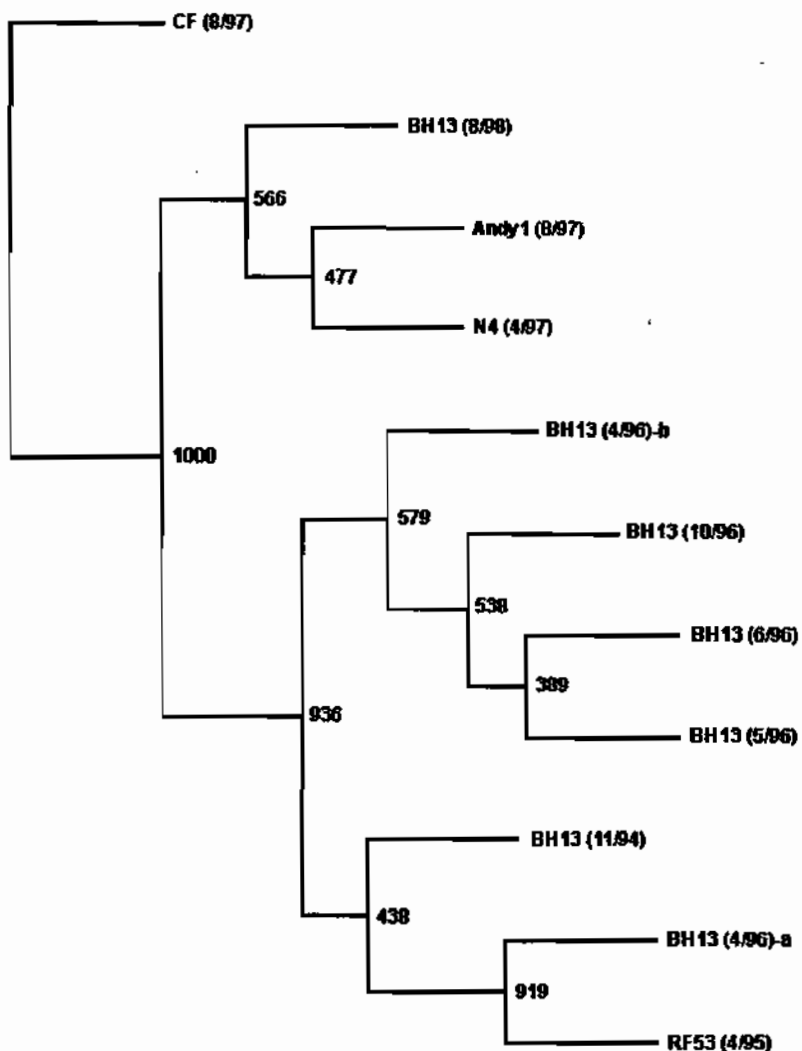


Fig. 2. Rooted Parsimony cladogram of the relationships of COII nucleotide sequences among collections of *Reticulitermes* spp. made over time from the BH13 inspection port on Sapelo Island, Georgia U.S.A. was constructed using PHYLIP programs (20); The data were bootstrapped 1000 times, then DNAPARS to infer trees and CONSENSE to get consensus tree (20). Figure drawn with TREEVIEW PPC (21).

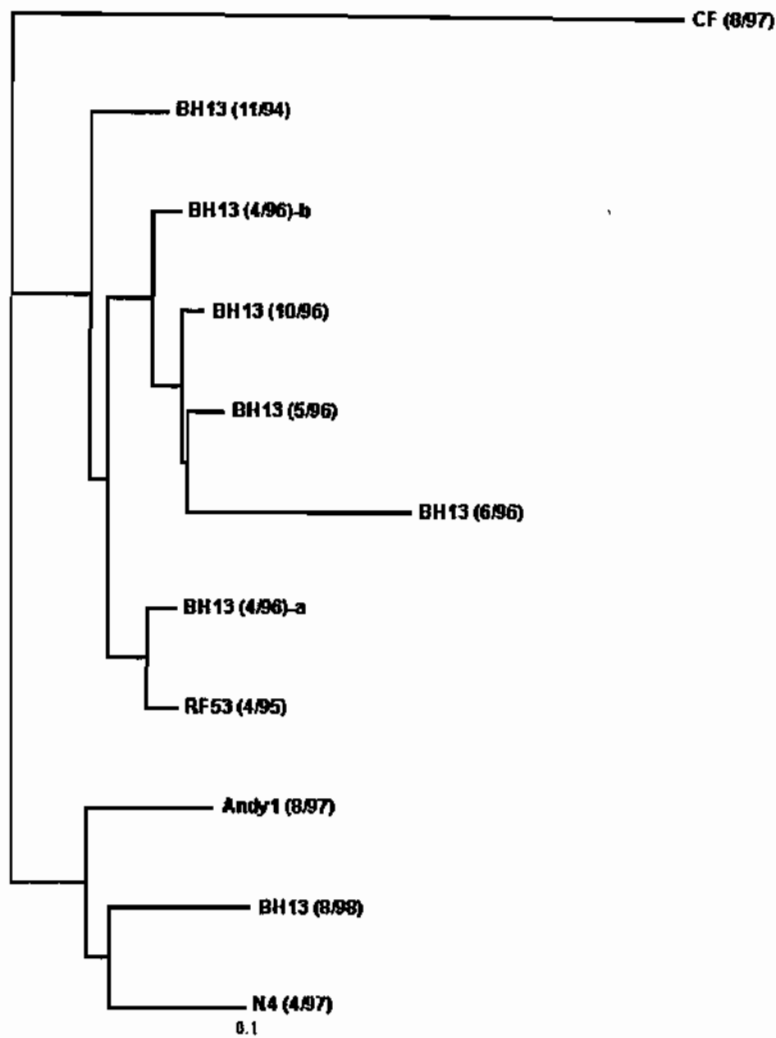


Fig. 3. Rooted Maximum Likelihood cladogram using the same data set as Figs. 1 and 2. The tree is not bootstrapped and was constructed DNAML from PHYLIP (20). Tree drawn with TREEVIEW PPC (21).

genotypes supported by bootstrap values (Figs. 1, 2). Further, all three trees similarly group BH13(4/96)-a and BH13(4/96)-b.

The COII analyses in the BH13(4/96) collection indicate the presence of at least two different female genotypes, demonstrative of a primary polygyne colony. Although primary polygyny has been demonstrated in

other termite families (Darlington 1985; Thompson and Hebert 1998). This is the first time that mtDNA data has been used to suggest it in *Reticulitermes*. Molecular differences recorded for the BH13 inspection port collections from 1996, when we had assumed a single colony of one species was visiting the site, can be evaluated in light of the following scenarios:

- a single polygynous or meta-colony organization displaying a kin-biased foraging strategy
- five different colonies, of the same species, foraged at inspection port BH13 over an 8 month period in 1996, two colonies on the same date in April, and one colony each on May, June, and November

Data obtained using a non-specific multilocus DNA fingerprinting technique has previously provided evidence for kin-biased foraging in termites of a polygynous, nest building, surface foraging African Rhinotermitid from the genus *Schedorhinotermes* (Kaib *et al.* 1996). If *Reticulitermes* exhibit polygyny and kin biased foraging it would provide an explanation for our BH13 1996 observations. In addition to the data presented in this report we have, to date, examined COII sequence from 73 individual termites from 20 different inspection ports across three Georgia soil provinces. All were single date collections. Two other inspection ports have, thus far, provided evidence of more than one maternal lineage within termites collected on the same date. Therefore, one maternal lineage per collection should not be assumed for *Reticulitermes*.

In addition to published observations (Pickers 1934; Howard and Haverty 1980), anecdotal evidence highlighting the dynamic nature of subterranean termite reproductive strategies has been provided by our field biology studies. We have, over the past five years, periodically captured primary reproductive pairs, numerous neoteric reproductives, and combinations of primary and neoteric reproductives from single, separate inspection ports. It is likely, therefore, that additional evidence of multi-maternal lineage groups will be discovered as we examine more individuals. Further, theories of *Reticulitermes* colony composition range from closed societies centered around a single pair of reproductives to open societies where intraspecific groups share resources (Clement 1986). Most of the evidence for intraspecific resource sharing in *Reticulitermes* has come from behavioral assays (Clement 1986; Thorne and Haverty 1991). Based on the concept of kin recognition, behavioral assays accept displays, or lack thereof, of overt aggressive behavior as an indicator of colony structure. However, we

recently demonstrated that peaceful cohabitation is possible between described species when aggressive individuals are removed from interspecific groups (Polizzi and Forschler in press). The complex behavioral responses displayed by these insects therefore suggest that behavioral assays alone cannot, with certainty, provide definitive determination of colony associations.

Our genetic and behavioral data suggest the possibility of coalescence and/or pleometry in *Reticulitermes* spp. eusocial behavior and, therefore, multimaternal (meta) colony composition. Budding is generally accepted as a mechanism for new colony formation in *Reticulitermes* based on the monogyne, kin based society concept. The idea of coalescence and/or pleometric colony formation, in which maternally distinct groups come together in a cooperative fashion, has been demonstrated in some termite genera (Pickers 1934; Darlington 1985; Thorne 1985) but never verified for subterranean termites using DNA sequence. Our BH13 data is, therefore, the first molecular DNA examination of individuals from a single subterranean termite feeding site over time and is compatible with a single colony being formed as a result of the fusion of as many as five maternal colonies. We show that a single feeding site can be occupied at a specific time and over time by different mtDNA genotypes, which means that the monogyne colony concept for *Reticulitermes* spp. (Isoptera: Rhinotermitidae) can no longer be assumed. Further, basing experimental designs on monogyny and extracting DNA from groups of termites (Broughton and Grace 1994; Broughton 1995) can mask variation. DNA extraction for population genetics studies, therefore, should be done on individual termites.

Intraspecific cooperation or competition in resource utilization could also explain these results. MRR studies have assumed that, unless indicated by morphometric characters, consistent visitation to an inspection port was the result of single colony habitation (Su *et al.* 1993; Forschler 1996; Forschler and Townsend 1996). Our MRR field research has suggested relatively small colony sizes compared to some of the published literature (Grace *et al.* 1989; Su *et al.* 1993). The Reynolds Mansion, Sapelo Island study site, for example, has 5 MRR-defined colonies in 24 of the 35 inspection ports. The remaining 11 inspection ports, including BH13, have not been associated using MRR to subterranean termites from another inspection port. It is possible that each of the 11 unassociated inspection ports is occupied by separate monogyne colonies whose movement between inspection ports occurs on a monthly, or shorter, schedule. If so, then the mobility of *Reticulitermes*

colonies is greater than previously assumed using MRR methodologies. Our data also argues strongly that changes in colony movement and resource utilization patterns cannot be elucidated using traditional nonmolecular techniques alone.

The COII sequence data demonstrates, regardless of which scenario proves more correct, that DNA markers are particularly well suited for the study of subterranean termite colony structure and are likely to be more illuminating than MRR, morphometric, and behavior studies alone. Further, *Reticulitermes* natural history will have to be reevaluated at the level of evolution of eusocial behavior, colony organization and development, colony movement and competition for resources, and evaluation of population management control tactics.

REFERENCES

- Altschul, S. F. et al. Basic local alignment search tool. *J. Mol. Evol.*, 215, 403-410 (1990).
- Avise, J. C. *Molecular markers, Natural History and Evolution*. (Chapman & Hall, New York, 1994).
- Broughton, R. E. & Grace, J. K. Lack of mitochondrial DNA variation in an introduced population of the Formosan subterranean termite (Isoptera: Rhinotermitidae). *Sociobiology* 24, 121-127 (1994).
- Broughton, R. E. Mitochondrial DNA variation within and among species of termites in the genus *Zootermopsis* (Isoptera: Termopsidae). *Ann. Entomol. Soc. Am.* 88, 120-128 (1995).
- Clément, J. L. Open and closed societies in *Reticulitermes* termites (Isoptera: Rhinotermitidae): Geographic and seasonal variations. *Sociobiology*, 11, 311-323 (1986).
- Darlington, J.P.E.C. Multiple primary reproductives in the termite *Macrotermes michaelseni* (Sjostedt). *Caste Differentiation in Social Insects*. (Eds. J.A.C. Watson, B.M. OkotKotber, CH. Noirot) (Pergamon Press, New York, 1985).
- Felsenstein, J. *PHYLIP (Phylogeny Inference Package) Manual* (University of Washington, Seattle 1993). Distributed by the Author.
- Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783-791 (1985).
- Forschler, B. T. & Townsend, M. L. Mark-release-recapture estimates of *Reticulitermes* spp. (Isoptera: Rhinotermitidae). *Environ. Entomol.* 25, 952-962 (1996).
- Forschler, B. T. Baiting *Reticulitermes* (Isoptera: Rhinotermitidae) field colonies with abamectin and zinc borate-treated cellulose in Georgia. *Sociobiology* 28, 459-484 (1996).
- Grace, J. K., Abdallay, A. & Farr, K. R. Eastern subterranean termite (Isoptera: Rhinotermitidae) foraging territories and populations in Toronto. *Can. Entomol.* 122, 551-556 (1989).
- Haverty, M. I., Forschler, B.T., & Nelson, L. J. An assessment of the taxonomy of *Reticulitermes* (Isoptera: Rhinotermitidae) from the southeastern United

- States based on cuticular hydrocarbons. *Sociobiology* 28, 287-318 (1996).
- Hein, J. A new method that simultaneously aligns and reconstructs ancestral sequences for any number of homologous sequences, when the phylogeny is given. *Mol. Biol. Evol.* 6, 649-658 (1989).
- Howard, R.W. & Haverty, M. I. Reproductives in mature colonies of *Reticulitermes flavipes*: abundance, sex-ratio, and association with soldiers. *Environ. Entomol.* 9, 458-460 (1980)
- Jenkins, T. M., C.J. Basten, Robin Dean, S.E. Mitchell, S. Kresovich, & B.T. Forschler 1999. Matriarchal genetic structure of *Reticulitermes* (Isoptera: Rhinotermitidae) populations. *Sociobiology* 33(3): 239-263
- Kalb, M., Hussenender, C., Epplen, C., Epplen, J. T. & Brandl, R. Kin-biased foraging in a termite. *Proc. R. Soc. Lond.* 263, 1567-1532 (1996).
- Liu, H & Beckenbach, A. T. Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol. Phylogenet. Evol.* 41, 41-52 (1992).
- Page, R. D. M. An application to display phylogenetic trees on personal computers. *Comp. Applicat. Biosci* 12, 357-358 (1996).
- Pickens, A. L. The biology and economic significance of the western subterranean termite, *Reticulitermes hesperus*. In *Termites and Termite Control* (ed. C. A. Kofoid) (University of California Press, Berkeley, CA, 1934).
- Polizzi, J. M. & Forschler, B. T.. Intra- and interspecific agonism in *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) and effects of arena and group size in laboratory assays. *Insectes Soc.* 45, 43- (1998).
- Polizzi, J. M. & Forschler, B. T.. Factors affecting aggression among the worker caste of *Reticulitermes* spp. subterranean termites (Isoptera: Rhinotermitidae) *J. Insect Behavior*, in press.
- Su, N.-Y. & Scheffrahn, R. H. A review of subterranean termite control practices and prospects for integrated pest management programs. *Integrated Pest Mgt. Reviews* 3, 113 (1998).
- Su, N.-Y., Ban, P. M. & Scheffrahn, R. H. Foraging populations and territories of the eastern subterranean termite (Isoptera: Rhinotermitidae) in southeastern Florida. *Environ. Entomol.* 22, 1113-1117 (1993).
- Thorne, B. L. Biology of subterranean termites of the genus *Reticulitermes*. In *NPCA Research Report on Subterranean Termites*. (NPCA, Dunn Loring, Virginia, 1998).
- Thorne, B. L. 1985. Termite polygyny: the ecological dynamics of queen mutualism. In *Experimental Behavioral Ecology* (eds. B. Holldobler and M. Lindauer), pp. 325-41. G. Fisher Verlag, Stuttgart.
- Thorne, B.L. & Haverty, M. L. A review of intracolony, intraspecific, and interspecific agonism in termites. *Sociobiology* 19, 115-145 (1991).
- Thorne, B. L., Russek-Cohen, E., Forschler, B. T., Breisch, N. L. & Traniello, J. F. A. Evaluation of mark-release-recapture methods for estimating forager population size of subterranean termite (Isoptera: Rhinotermitidae) colonies. *Environ. Entomol.* 25, 938-951 (1996).

