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Evaluation of subterranean termite biology using genetic, chemotaxonomic, and morphometric markers and ecological data: a testimonial for multi-disciplinary efforts

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ABSTRACT

Two main problems confront researchers attempting to study the biology of subterranean termites from the genus *Reticulitermes*. The current taxonomic scheme needs revision. Equivocal identification of specimens using published dichotomous keys often complicates field and laboratory tests. In addition, a realistic definition of what constitutes a subterranean termite colony elevates the role of inference to the status of dogma. In this paper we outline both problems and propose a multidisciplinary approach using behavioral, ecological, chemotaxonomic, and genetic data that promises to resolve both dilemmas.

INTRODUCTION

Subterranean termites of the genus *Reticulitermes* are ecologically important as decomposers of cellulose. Yet because they are responsible for approximately one billion dollars in structural damage and control costs each year in the United States alone, it is their economic impact that has likely stimulated extensive study of their biology for most of the 20th century [1,2,3]. Despite the fact that *Reticulitermes* species have considerable economic and ecological impact, there remain many unanswered or ambiguously answered questions concerning their biology. The crytobiotic, eusocial behavior of these small, soft-bodied, diplo-diploid termites has made them difficult to study. Even the terminology used to describe their social castes and those behaviors important in control tactics generates confusion among readers [4,5]. Research is also hampered by not being able to consistently identify specimens to species. This is due in part because the dichotomous keys used for identifying *Reticulitermes* species are based on morphological characters of the least abundant caste (alates and/or soldiers) [6,7,8,9]. Alates are found seasonally and soldiers comprise only 1-2% of the population [1,2,7]. Species determination, therefore, may be equivocal if neither or only one of these castes is found [7,10,11].

Subterranean termite field research involves collecting data at specific time intervals from disparate locations that are concealed from view. Data analysis is, therefore, often assumption driven and open to multiple interpretations. Conventional field research techniques detect the presence of termite activity by the bait stake method [12]. Following detection of activity, monitoring stations or inspection ports that contain a food and/or aggregation substrate are put into the ground or placed on the soil surface for the purpose of assembling these cryptic insects at known sites [13]. Visitation to inspection ports by subterranean termites is then recorded on scheduled collecting appointments (weekly, bimonthly, monthly). Information collected could include counts of individuals, live weights, caste proportions, morphometric species identification, and feeding rates (wood, paper, and cardboard consumption). Additionally, mark-release-recapture (MRR) techniques and agonism bioassays have been utilized to determine related use of inspection ports within a given area [14,15,16].

Temporal discrete data is collected from termite inspection ports. What has occurred during the intervening time interval is, by necessity, inferred. This makes it problematic to accurately determine population size, home range or foraging area, and consumption rates, as well as movement between or continuous use of inspection ports by the same termite population. Two assumptions are routinely made that influence data interpretation. First, that termite movement to and from inspection ports is random, resulting in equivalent distribution of individuals between feeding sites [17]. Second, that the same population consistently visits a specific inspection port, unless a change in live weights or morphology indicates otherwise [18]. It has proved misleading, however, to determine colony relationships solely on MRR evidence, as well as to assume continuous use of an inspection port by the same population using MRR and morphology alone [19]. Subterranean termite colony structure and species composition, therefore, appear to be too elusive for traditional field techniques alone to decipher beyond the perpetuation of old assumptions.

Two main problems emerge in the study of subterranean termites: lack of taxonomic clarity as to species designation, and a pragmatic definition of a subterranean termite colony - both of which cannot be resolved with traditional field techniques. We have collected alate and soldier caste members from a single termite inspection port that, using published taxonomic keys, keyed to two different species, depending on the caste used for the identification [10]. Additionally, our work with MRR methodologies and DNA markers has raised questions concerning colony structure and movement between established feeding sites [19,20]. It is our contention, therefore, that a combination of scientific methodologies to include traditional field techniques and morphological, chemical, and genetic characters would better elucidate both the taxonomy and colony structure of *Reticulitermes* species.

The objective of this paper is to present the scientific techniques that have been employed in the study of subterranean termites from the genus *Reticulitermes*. We will discuss the implications of the data that we have collected with these techniques and propose that a multi-disciplinary approach should lead to a better-than-pragmatic understanding of subterranean termite biology.

THE SPECIES QUESTION

Unambiguous species identification is imperative for understanding both the ecological role and behavior variation of an animal. In 1920, Banks and Snyder published the first classical morphometric descriptions of *Reticulitermes* species found in the United States, based on two terminal castes - the alate or soldier [21]. Several lists of species and/or keys have been published since [7,8,9,22,23,24,25,26,27]. We believe, however, these represent no revision of that first taxonomic scheme [10]. As mentioned, we have found subterranean termites at the same feeding site or inspection port that key to two different species depending on the caste used in identification [10]. We concur, therefore, with the observations of other contemporary workers in the field [8,9,28] that the taxonomy of the genus *Reticulitermes* needs revision. Further, recent work using genetic, chemotaxonomic, and behavioral characters [10,11,16,19,29,30] corroborates this need as it reinforces the value of an integrated approach to species identification.

Two chemotaxonomic characters, cuticular hydrocarbons (CH) and terpenes, have provided alternate, corollary measures to morphometric descriptions. CH profiles over disparate locations have proven repeatable [29,31] and, therefore, have been used to suggest new *Reticulitermes* species [10,31,32]. Furthermore, CH phenotypes have correlated well with DNA genoytpes (33) indicating the feasibility of using the two markers together. Terpenes, a constituent of subterranean termite soldier defense secretions, have unique chemical phenotypes, which has also made them useful as species markers [34,35]. Both of the aforementioned phenotypic characters demonstrate the potential of using chemotaxonomic techniques for evaluating species-specific differences as well as for integrating these phenotypes with morphometric characters and DNA genotypes.

Behavioral data has been collected and interpreted in an attempt to distinguish species-specific characters for *Reticulitermes*. Displays of overt aggression or agonism between species have been recorded in bioassay using a variety of termite species [36]. Observations, however, of agonistic behavior have proven equivocal with species studied in the southeastern United States. The worker caste from two sympatric species, *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks), were used in a bioassay

designed to test agnostic behavior between species [30]. It was observed that inter-specific groupings showed as much aggression (or lack of) as intra-specific groupings. The evidence also suggested that numerous factors, including the physiological condition of the termite tested, resulted in a division of labor involving display of aggression [37]. This presents a complicating variable to using agonism bioassays alone as a species separation tool. Behavioral assays recording overt aggressive behavior may be a useful tool with certain species pairs. If no aggression is noted, however, then the results should be analyzed with caution. More subtle behavioral cues indicative of kin or species recognition may provide more consistent behavioral data [38,39], and, thereby, be a better species-specific character. Such kin recognition behaviors should be pursued further.

Additional tools are offered by biotechnology. Recovery of genetic markers has been shown to have import for penetrating taxonomic questions. Direct DNA sequencing of a 16S ribosomal RNA gene fragment and the cytochrome oxidase II (COII) gene have been used to determine the phylogenetic relationships among termite families and subfamilies [40,41]. But, until recently, no direct DNA sequencing had been used to distinguish between *Reticulitermes* species.

Sequences from the internal transcribed spacer (ITS) regions of the multicopy nuclear ribosomal DNA (rDNA) have been shown to evolve faster than nuclear coding regions [42]. They have been exploited in entomology as species markers [42,43,44,45] for intra- and interspecific discrimination [44,46,47]. We, therefore, used the polymerase chain reaction (PCR) and conserved insect primers [43] to amplify ITS2 fragments from subterranean termites collected from several soil provinces in the state of Georgia, USA. These fragments were sequenced [48] and evaluated using phylogeny analyses [19]. Three sympatric *Reticulitermes* species were differentiated along morphometrically determined species lines, but intraspecific variation was not significant. Thus, while not useful at the intraspecific level, ITS2 sequence data may differentiate at the interspecific level. In addition, we have successfully used mitochondrial DNA (mtDNA) sequence data to differentiate between sympatric *Reticulitermes* species while examining intra-specific differences [11,19]. This work has also indicated the possibility of new taxa [11,19].

Determination of the status of one species, *R. flavipes*, is a case that speaks to the problems surrounding the taxonomy of the genus. This species was first described from specimens collected in a glasshouse in Vienna, Austria, in 1834 [49]. The type specimen, therefore, for the most common subterranean termite found east of the Mississippi River in the United States is still held in Europe. Recently we completed phylogenetic analyses of two mitochondrial DNA (mtDNA) genes and the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS2) from specimens representing 16 *Reticulitermes* populations from the United Kingdom, France, and the United States. Our analyses reveal that *R. flavipes* from Georgia, USA, is closely related to the European species *R. santonesis*. Reports from France indicate the possibility that *R. santonesis* could indeed be *R. flavipes* [50]. Additionally, we observed that *R. speratus* from Japan appears to be very closely related to *R. lucifugus* in Europe. Is it possible that *R. lucifugus* was transported to Japan from Europe as part of maritime commerce or visa versa? Further, DNA sequence analyses may not only help provide taxonomic clarity, but illuminate gene flow patterns and relationships among the Asian, European, and American populations.

It is apparent that currently used morphometric characters cannot, alone, consistently delineate *Reticulitermes* species. Although genetics, behavioral, and chemical techniques each provide informative species characters, one specific technique should not be used exclusively to delineate the species of *Reticulitermes* in the United States. Recently we found that the correlation among morphometric characters, mtDNA sequence genotypes, and CH phenotypes demonstrated good agreement for the three sympatric species described in Georgia, USA [33]. The study also demonstrated that a group in which morphometric characters for alates and soldiers each keyed to a different species is likely a new taxon [33]. We propose, therefore, that combinations of these techniques have the power to successfully differentiate species and that a multidisciplinary approach should be undertaken to revise the genus.

THE COLONY QUESTION

A working definition of a social insect colony has been provided in numerous texts, but, in general, it can be defined as that group of individuals who cooperate in

exploiting a resource(s) and share in rearing future generations [51]. This definition would be acceptable to most termite biologists. Determination, however, of which termites found at disparate locations during field research are actually involved in "colony" activities is difficult. It is generally agreed that subterranean termite colonies occupy one or more feeding sites, which they locate, excavate, and then inhabit. These feeding sites are interconnected by a diffuse network of underground or covered (shelter tube) tunnels. Movement of termites toward locating feeding sites, establishing (colonizing) feeding sites and, then, movement between established feeding sites can be discerned only through the temporal collection of termites from inspection ports or by directed or serendipitous collections of woody debris. However, numerous factors, including the size of the population, availability of alternate food resources, climatic conditions, quality of the food substrate, competition with other termite populations, and disturbance caused by collecting contribute to termite visitation to inspection ports, yet are often not acknowledged [20].

Traditionally, termites found during field studies are identified as colony units using MRR methodologies in combination with comparison of morphometric characters and live weights of the insects collected at a site. In general, termites are collected at an inspection port and marked either in the field with a topical mark or returned to the laboratory where they are stained (marked) by feeding termites a dye-impregnated substrate [14,15,52,53]. These marked termites are then released back into the inspection port from whence they were originally collected. After a prescribed time period, all of the inspection ports within the same general area are sampled for marked termites. Those inspection ports that contain marked termites are then considered 'connected' and assumed to represent a colony unit. Often the process is continued, such that those inspection ports that contain marked termites after one mark-release-recapture cycle are also marked and released. The function of MRR technique(s), therefore, is to track the movement of marked individuals and to extrapolate from this movement the relationship between 'adjacent' inspection ports. The recovery of marked termites from a site where no marked termites were released, therefore, is considered to demonstrate a relationship indicative of a single subterranean termite colony based, largely on the assumption of random movement between inspection ports.

Our field data has led us to question the assumption of random movement of termites within a population, and therefore, equivalent distribution between inspection ports. The information recorded during subterranean termite field studies is often reported using the combined data from all inspection ports identified as a colony unit [54,55,56]. Examination of feeding rates or the number of termites collected by inspection port, however, can provide more information about the patterns of termite movement between established feeding sites. Figure 1 shows the physical disposition of 7 inspection ports 'connected' by MRR techniques at one of our field sites located at the northwest corner of the Reynolds Mansion on Sapelo Island, Georgia. Figure 2 shows the wood consumption, number of termites collected, and soldier proportions for that 'colony' (all 7 inspection ports combined), by month, over a one year period. The combined data for wood consumption rates, considered the most 'continuous' measure of activity, collected from these MRR connected inspection ports reveals a reasonable seasonal pattern - one approaching a Poisson

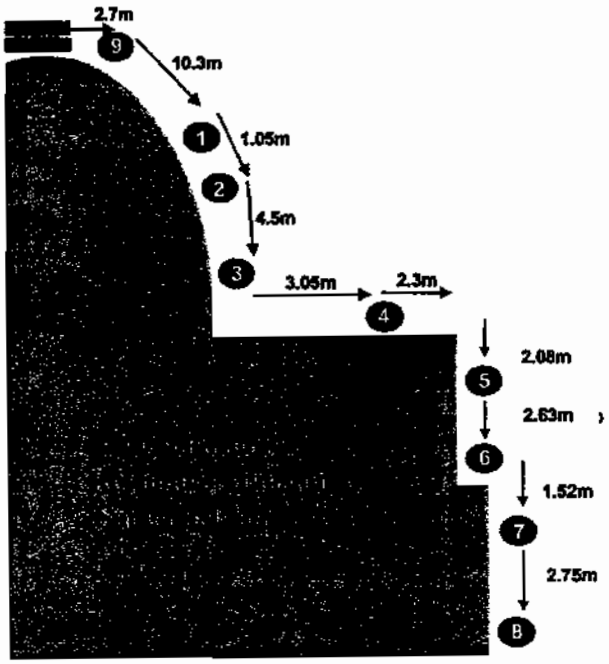


Figure 1. Physical arrangement of inspection ports identified by MRR as used by the same subterranean termite population indicative of a colony unit.

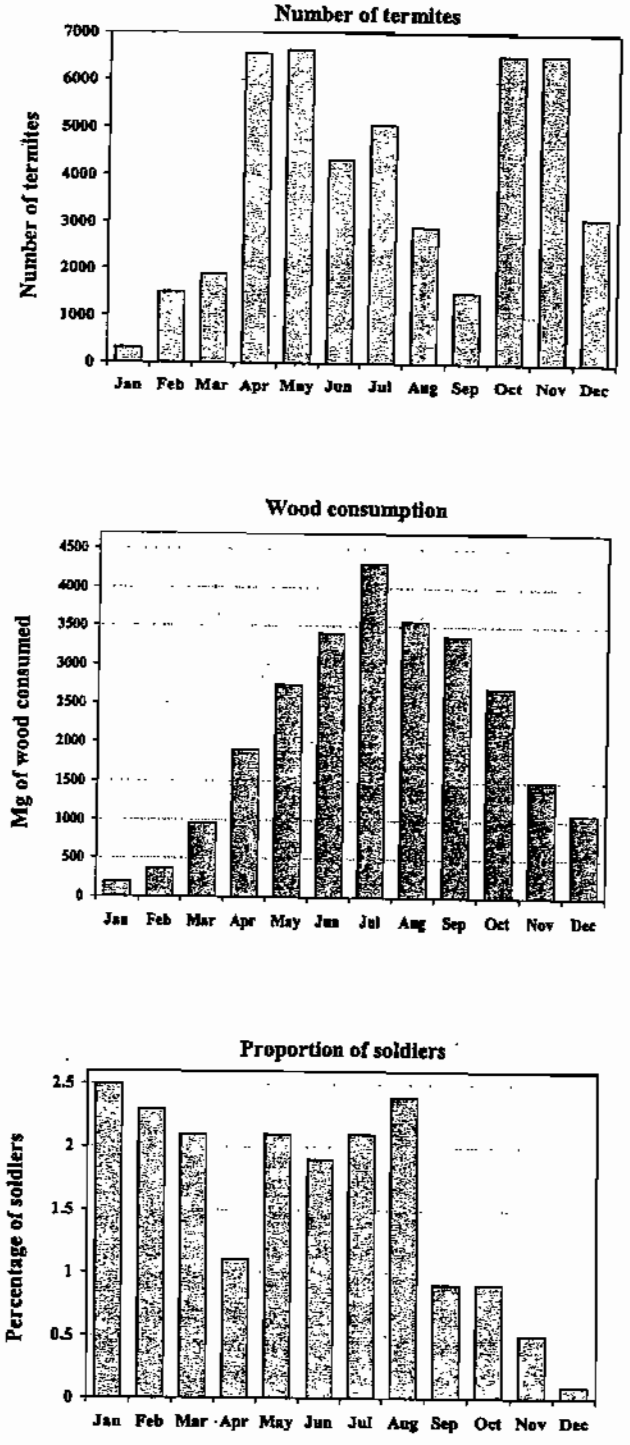
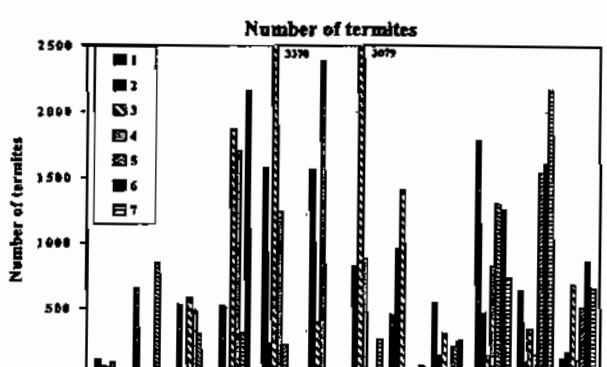
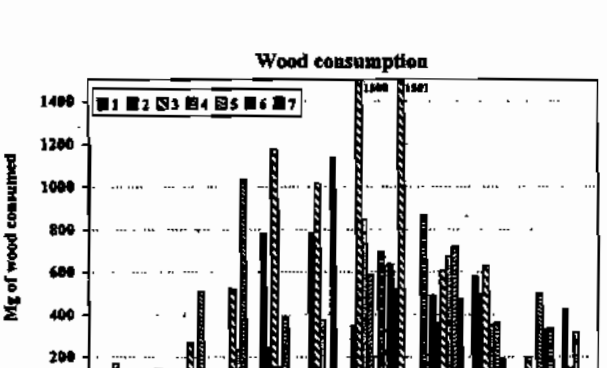


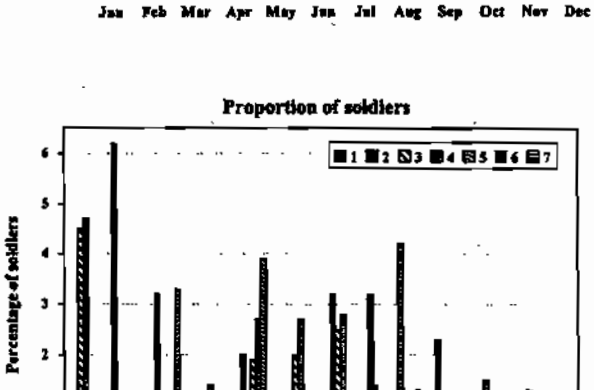
Figure 2. Measures of subterranean termite activity combining data collected from seven inspection ports identified as a colony using MRR.

Distribution - and assumes consistent visitation at all 7 available, known feeding sites (inspection ports). The number of termites collected, considered a more temporal view of subterranean termite activity, demonstrate a level of activity more sensitive to local weather conditions (fewer termites collected during the warmer, dryer summer months). Figure 3 shows the feeding rate, number of termites collected and soldier proportions by inspection port for this same 'colony' over the same one year period by individual inspection port (aligned by month in sequence to the physical disposition in Figure 1). When these measures of termite activity are examined by inspection port, however, we see a clumped distribution. Although the combined data for the proportion of soldiers collected indicate a slight decrease during the winter months (from 2% to >1%), the data also indicate a clumped distribution when examined by inspection port (range 0-6.2%) (Figures 2 and 3). The same clumped distribution is clear in the data concerning the recovery of marked termites over time.

We released 1200 termites stained with the relatively persistent, fat-soluble dye Nile Blue A into one inspection port, of the same 'colony' mentioned above (inspection port #5). These data, collected one year after the aforementioned activity data were collected, includes two additional inspection ports – numbers 8 and 9 located (physically) outside the previously identified foraging range. This single release of marked termites was conducted in the fall. The appearance (or lack thereof) of marked termites was then recorded for 7 consecutive months. Figure 4 shows that the number of marked termites was higher in the inspection port (#6) next to the site of release one month after release. Two months after release, the number of marked termites was equivalent between two inspection ports (#'s 3 & 6) on either side of the release site, although one inspection port (#4), between the aforementioned sites, provided no marked termites. For the third through fifth months after release, the number of marked termites captured was consistently higher in an inspection port (#7) two sites removed from the release site. Indeed, although marked termites were eventually found in each inspection port, we did not find marked termites in all of the 9 inspection ports in the same month. Throughout the entire study, at least one or more of the inspection ports provided no marked termites (2 at one and two months, 1 at three months, and 4 at four and five months) when and where unmarked termites were

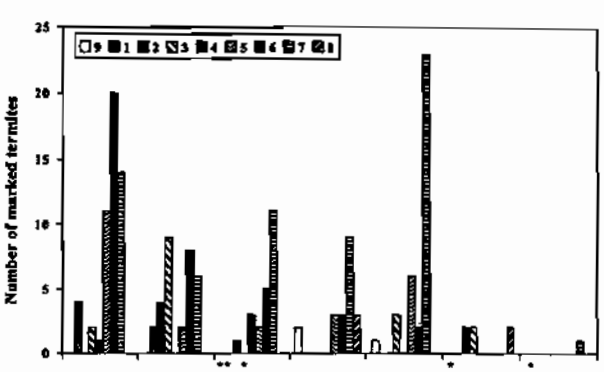






[1] Within each month inspection ports are arranged according to physical proximity in the field.
* Indicate that no termites were collected.

Figure 3. Measures of subterranean termite activity by individual inspection port from seven inspection ports identified as a colony using MRR.[1]



[1] Within each month inspection ports are arranged according to physical proximity in the field.
* Indicate that no termites were collected.

Figure 4. Number of marked subterranean termites recaptured by individual inspection port from nine inspection ports identified as a colony using MRR.[1]

collected. The data collected from MRR studies is temporal in nature, as our results in Figures 3 and 4 demonstrate. Random distribution of subterranean termites within populations and between inspection ports, therefore, cannot be assumed when interpreting field data collected from MRR studies.

Although the estimation of termite population size using MRR has come under criticism [13,57,58] the question of using MRR techniques to identify "colony" relationships has not been thoroughly examined. The appearance of one marked individual in an inspection port has been used to identify the relatedness or common use of inspection ports by a 'colony' of subterranean termites. It has also been the justification for then marking all the termites found within the additional inspection port for further delineation of colony associations. Our fieldwork with less persistent marks (i.e., fluorescent spray paint) has indicated the potential for overestimating subterranean termite colony associations using long-term marks (fat-soluble dyes). In a recent study, we demonstrated that the collection of one marked termite in a separate inspection port (non-release site) did not indicate use of that feeding site by a single termite population [20]. In that particular study, use of baits confirmed the lack of connection between the inspection ports in question.

Additional corroborating evidence indicating the potential for subterranean termite populations sharing individuals (between colonies) has been secured using a mtDNA marker [19] and alloenzyme makers [59,60]. Therefore, MRR data must be interpreted with caution and should not be the sole measure of colony relationships.

Indirect measures of genetic variation have been used with success to examine questions of *Reticulitermes* population dynamics although direct knowledge of DNA sequence data would provide the ultimate information. Non-denatured proteins are charged nuclear markers. Protein electrophoresis and the allozyme variants revealed in the zymogram patterns produced can, therefore, be interpreted in terms of Mendelian genotypes and analyzed for population partitioning with Wright's F-statistics [61]. Even though allelic variation can be hidden unless sequential electrophoresis [62] is employed, these assays have revealed gene expression patterns that encompass the natural history or evolution and gene flow of *Reticulitermes* species [59,61].

The paradigm is that *Reticulitermes* colonies are monogyne – founded by a single alate pair following a flight or through budding where secondary reproductives (progeny of the original founding queen) establish separate centers of activity [2,28]. The monogyne colony hypothesis permits the assumption that all colony progeny would inherit the maternal lineage of the original founding female. Mitochondrial DNA is a nonrecombining, haploid molecule inherited maternally, meaning from the female colony progenitor. Thus each colony should have a specific mtDNA genotype inherited from the colony matriarch. This colony specific mtDNA genotype also means that movements of individual colony members can be monitored and/or verified over time with the DNA marker.

We used mtDNA sequence to test the assumption that a single species from a single colony would be collected from an individual inspection port over time. Individuals were collected from an inspection port, designated BH13, on consecutive months, 4/96, 5/96, 6/96, then 10/96 and from the years 1994, 1996, 1998 [19]. DNA was then extracted [11] from individuals collected from the BH13 inspection port. The mitochondrial cytochrome oxidase subunit II (COII) gene was amplified by PCR and sequenced [19] from each individual collected. The mtDNA genotypes were compared and subjected to phylogeny analyses [19]. Figure 5 clearly shows that individual mtDNA genotypes varied by month, year, and sometimes within the same collection date, as the two different mtDNA genotypes found in the BH13 collection of 4/96 show. Although primary polygny has been demonstrated in other termite families [63], this was the first time that mtDNA genotypes were used to suggest it in *Reticulitermes*. Further, the different mtDNA genotypes from 4/96, 5/96, 6/96, and 10/96 (Figure 5) are particularly interesting, since a single colony of one species was the assumption based on the morphological homology of the termites collected during that timeframe. There could be two explanations for the data variation in genotype. First, "a single polygnous or meta-colony organization with a kin-biased foraging strategy" [19] could account for the observation. Second, "five different colonies, of the same species, could have 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" [19]. But, regardless of which

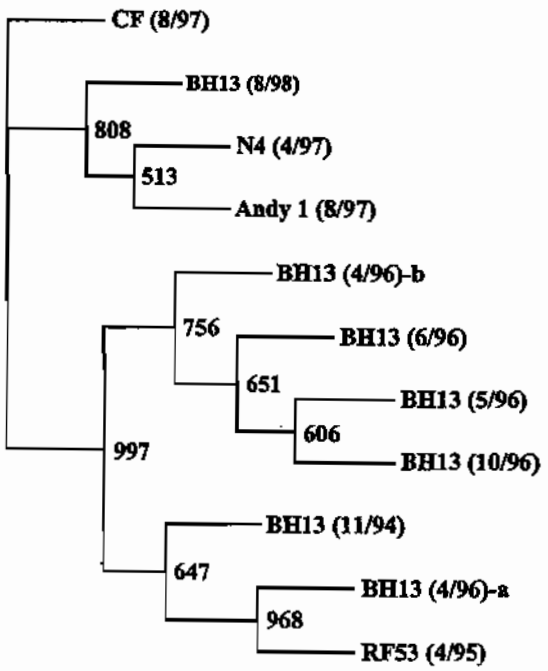


Figure 5. Rooted Neighbor Joining (NJ) phylogram of published COII gene sequence data [19].

explanation, if either, is responsible for our observations, it is clear that DNA markers provide insights that MRR, morphology, and behavior studies alone do not. Amplified fragment length polymorphism (AFLP) is the most recent biotechnology to be used in our laboratory. Preliminary data show that the AFLP fingerprint is species, population and individual specific. We plan, therefore, to inculcate AFLP fingerprint analysis into our multidisciplinary 'toolbox' in order to better evaluate subterranean termite population structure.

CONCLUSIONS

The taxonomy of the genus *Reticulitermes* needs revision to provide for continuity between research projects and to better understand the biology of sympatric species. We propose, therefore, a multi-disciplinary taxonomic study to include a major collaborative effort involving researchers using morphometric, chemotaxonomic, behavioral, and genetic data. This organismal approach should provide for a more complete and less equivocal definition of *Reticulitermes* species.

Field studies are hampered by not having a clear understanding of subterranean termite colony structure. It is imperative that we have a more substantive definition of a colony than what is available today. Attempts to delineate a colony in field studies should understand the temporal nature of the data collected using traditional entomological techniques. The study of the genus *Reticulitermes*, therefore, would be best served if molecular genetic technology in addition to chemical and morphometric measurements were employed in conjunction with field techniques. Each method of study alone presents a myopic view of the organism. We have demonstrated, however, that collaborative efforts involving multiple scientific methodologies not only provide the data and perspective to question old paradigms, but can bring us closer to understanding the organism and its ecological place.

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