AN INVESTIGATION OF THE INFLUENCE OF *Trichoderma virens* (HYPOCREALES: HYPOCREACEAE) ON *Reticulitermes virginicus* (ISOPTERA: RHINOTERMITIDAE) FEEDING, WITH AN EVALUATION OF THE USE OF LABRAL MORPHOLOGY FOR IDENTIFICATION OF *Reticulitermes* FROM TEXAS

A Thesis

by

BRYAN P. HEINTSCHEL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2006

Major Subject: Entomology

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Approved by:

Chair of Committee,	Roger E. Gold
Committee Members,	Jimmy K. Olson
	Charles M. Kenerley
Head of Department,	Kevin Heinz

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ABSTRACT

An Investigation on the Influence of *Trichoderma virens* (Hypocreales: Hypocreaceae) on *Reticulitermes virginicus* (Isoptera: Rhinotermitidae) Feeding, with an Evaluation of the Use of Labral Morphology for Identification of *Reticulitermes* from Texas. (May 2006)

Bryan P. Heintschel, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Roger E. Gold

Subterranean termites encounter numerous kinds of fungi during foraging and feeding activities. Nearly nine decades of research have exposed only a small fraction of the termite-fungal interactions that exist in nature. The first portion of research presented here focused on how feeding behaviors of *Reticulitermes virginicus* (Banks) were affected by the fungus *Trichoderma virens* (Miller, Giddens & Foster) von Arx. Tests were performed with 'P' (GLT+) and 'Q' (GLT-) strains of *T. virens*. Both strains were applied to filter paper and wood disks cut from southern yellow pine and Sentricon® monitoring devices. The first bioassay assessed the termites' feeding responses to fungal extracts removed from liquid media on days 2 through 7, and again on day 15. Only the GLT+ extracts from days 6 and 7 inhibited termite feeding significantly from the controls (16% and 54% less area loss, respectively). Response to wood covered by live *T. virens* mycelia was tested in the second bioassay. No significant differences in termite consumption were seen between fungal strains, but both substantially reduced the area loss due to termite feeding of treated wood by an average of 35%. A vacuum impregnation system was used to inoculate wood disks with fungal homogenate in the third bioassay. The wood treated with either GLT+ or GLT- homogenates did not have significant differences in area

loss due to termite feeding. Overall, these results reiterated the plasticity that exists with termite-fungal relationships.

The second research topic addressed the applicability of labrum-based identification techniques to *Reticulitermes* Holmgren in Texas. Soldier labral morphology of four species, *R. flavipes* (Kollar), *R. hageni* (Banks), *R. tibialis* Banks, and *R. virginicus* (Banks), was evaluated as a character to separate species. Length and width measurements of five soldier labra were taken from each of the eight collection sites. These results were then judged against molecular analysis of the mtDNA 16S rRNA gene. Findings showed that labral shape was an unreliable diagnostic characteristic when comparing all species. A combination of length and length-to-width ratio successfully segregated all four *Reticulitermes* species. Comparison of a morphology-based dendogram to the phylogenetic analysis revealed a shared pattern between phenotypic and genotypic variations.

DEDICATION

This thesis is dedicated to my fiancée, Jill Millican, for whom I owe the deepest gratitude. She has been by my side through this entire journey, offering her steadfast support. She has shared with me everything from the monotony of counting and weighing termites to the late nights of studying and writing. I believe that the greatest asset I relied upon throughout my work has been the love and friendship shared between us.

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There are numerous individuals to whom I owe acknowledgement, but none deserve more recognition than the members of my committee: Drs. Roger E. Gold, Jimmy K. Olson, and Charles M. Kenerley. If not for the vast opportunities they provided to me, I would be in a position far different from the one I am in now.

Dr. Gold, I came to your lab with ambitions and aspirations; you have imparted in me the ability to fulfill all of them and more. You never expect more from a student than they can give, yet you always find more in them than they even knew existed. With your instruction, I have developed the confidence and knowledge necessary to guide me through any daunting circumstance I may one-day face. Dr. Olson, you have been a mentor to me in the truest sense. Your advice on science and, in general, life has always led me in the right direction. The straightforward, tell-it-like-it-is mentality of yours will certainly remain with me throughout the rest of my days. Dr. Kenerley, I would still be an entomologist lost in the world of mycology had you not been there to guide me. The laboratory access you granted me alone made this project feasible. I also have to say thank you for reminding me to view the situation from both sides of the coin, the optimistic and the realistic.

If ever there were a position on my committee for an honorary member, it would be filled by Dr. James W. Austin. The expertise he provides made the genetic analyses in this research possible. When I had suspicions about termite identification methods, he directed me to the source for my answers. He has also been a critical source of encouragement, always reminding me how valuable any novel piece of information is to science.

Everyone associated with the Center for Urban and Structural Entomology during my presence there deserves recognition. I would like to specially thank Dr. Mark Wright for sharing

his wealth of intelligence with me, and his unique angles of interpretation that constantly remind me not to overlook the simple solutions. I also owe Laura Nelson, Bryce Bushman, Chris Keefer, and Bill Summerlin my appreciation for their continued help throughout the office, laboratory, and field. Over the course of the past few years, I have had the opportunity to work with a magnificent group of graduate students: Kim Engler, Barry Furman, Grady Glenn, Molly Keck, Jason Meyers, Anne Narayanan, and Rachel Wynalda. Each one of these people has become an irreplaceable friend to me. I would also like to pay tribute to the late Dr. Harry N. Howell, Jr., who made an impression on me that will be matched by no one. He was a rare individual, who viewed life with such compassion that you could not help but share in his enthusiasm. Whether it was his intelligence, his passion, or just the stories he told, everyone seemed to be caught by Harry.

I must also recognize Suzanne Segner and Aric Wiest from the Department of Plant Pathology and Microbiology here at Texas A&M. They readily shared with me their expertise in the field of mycology. The laboratory skills I learned from them were a vital asset to my research.

In closing, I wish to say thank you to the contributors of the scholarships I received during my graduate school career. These awards include the Ivey Memorial Scholarship, Bill & Casey Clark Endowed Scholarship, Coastal Bend Pest Control Association Scholarship, Charles "Buddy" Glasse Memorial Scholarship, Clayton Wright Memorial Endowed Scholarship, and the Clay Stroope Memorial Endowed Scholarship. This financial support was critical to my educational opportunity; I will forever be indebted.

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CHAPTER I

GENERAL INTRODUCTION

Subterranean termites, especially those in the genus *Reticulitermes* Holmgren (Isoptera: Rhinotermitidae), are some of the more economically-important pest insects of human-made structures. The primary *Reticulitermes* species viewed as structural pests in the United States are *R. flavipes* (Kollar), *R. hesperus* (Banks), *R. tibialis* Banks, *R. hageni* (Banks), and *R. virginicus* (Banks) (Potter 2004). Since 1986, estimates of the annual cost directed toward the control and repair of termite damage in the United States alone has exceeded the billion dollar mark (Su and Scheffrahn 1990, Gold et al. 1993, Su 1993, 2002). Termite feeding can cause damage that ranges from mere aesthetic blemishes to that which compromise the structural integrity of buildings. This is dependent upon several factors, some of them being the section of the structure attacked, the length of time until the infestation is detected, and the abiotic conditions of the area infested with termites.

The degree of damage can also be compounded by the species of termite attacking the structure. In regard to native subterranean termites, an identity of the culprit species may be determined, but it is done so with reservation. The reason lies in the lack of consistent, interspecific morphological characteristics that can be used to reliably separate *Reticulitermes* species. The size and shape of morphological characteristics have been employed in many diagnostic keys for termite species identification in North America (Banks and Snyder 1920, Banks 1946, Weesner 1965, Nutting 1990, Scheffrahn and Su 1994, Hostettler et al. 1995).

This thesis follows the style and format of the Journal of Economic Entomology.

Termite taxonomy in Europe has followed a similar methodology by using morphometric data to identify species (Clément 1978, 1979; Clément et al. 1982, 2001). The disadvantage of these approaches is that their degree of accuracy can be geographically dependent. This means that a correct identification will likely be made only if the specimen in question and the type specimen of the description are from the same geographic cline.

A cline, by classical theory, is a gradual progression of speciation occurring in two phases (the smooth cline and the stepped cline) across a geographical area (Huxley 1938, 1939). This theory was later revised to incorporate a third phase (the broken cline) during which the division of the new species actually concludes (Salomon 2002). Altitude and latitude have both been shown to initiate progressive changes in physical characteristics within species of vertebrates and invertebrates. Marked differences in color, size, and body proportions between house sparrows (*Passer domesticus* L.) sampled in northern sites versus those sampled in southern areas indicated clinal variation within the species (Johnston and Selander 1964). In insects, changes in the wing morphology of *Drosophila subobscura* (Collin) (Diptera: Drosophilidae) (Huey et al. 2000) and overall body size in species of *Bothrometopus* and *Ectemnorhinus* (Coleoptera: Curculionidae) (Chown and Klok 2003) have been connected with geographic clines.

There is reason to believe that subterranean termites are subject to geographic clines, which could complicate morphological identification. In theory, size divergence could exist between the termites inhabiting a semi-tropical area of Florida, USA and a colony of the same species found in the woods of Ontario, Canada. Size variations in *Reticulitermes* samples collected in far smaller spatial areas have routinely been noted in the taxonomic literature (Banks and Snyder 1920, Banks 1946, Weesner 1965, Nutting 1990, Scheffrahn and Su 1994, Hostettler et al. 1995). Many of these notations express the size ranges or shape differences typical for the

key diagnostic features of each species. Not only does this increase the ambiguity when trying to identify one or two specimens, but it also lessens the level of objectivity. For example, the following couplet taken from Scheffrahn and Su (1994) separating the soldiers of *R. flavipes* from those of *R. virginicus* and *R. hageni* leaves room for subjective personal interpretation.

13 Pronotum width usually greater than 0.90 mm; head length with mandibles equal to or greater than 2.8 mm; points of mandibles, especially left, curved inward about 70-90º ……....…….*Reticulitermes flavipes*

\- Pronotum width usually less than 0.85 mm; head length with mandibles less than or equal to 2.7 mm; curvature of mandible points 45-90º..... ……..…….....….. 14 [leads to couplet for *R. virginicus* and *R. hageni*]

Hostettler et al. (1995) re-enforces the issue by reporting the presence of morphometric variation at the colony level. An overlap in the size range between two species inevitably occurs because of these natural variations. This leads to complexities and often inaccuracies when attempting to identify a specimen based solely on morphological traits (Haverty and Nelson 1997, Foster et al. 2004).

However, termite taxonomy has by no means remained stagnant. Advancements in biochemical, chemical, and molecular analyses have been increasingly applied to aspects of species identification of the last twenty-five years. Biochemical assessments of allozyme polymorphisms and diagnostic alleles in *Reticulitermes* have been successful at discerning species distinctions (Clément 1981, 1984; Clément et al. 2001). However, the practicality of these techniques is hindered by the fact that specimens must be from the same life stage and either alive or preserved by freezing at -80ºC. Several studies have since focused on the

chemistry of termite cuticular hydrocarbons for species taxonomy (*Reticulitermes*: Haverty et al. 1991, 1996a, 1999; Clément et al. 2001, *Coptotermes*: Haverty et al. 1991, *Nasutitermes*: Haverty et al. 1996b, *Odontotermes*: Kaib et al. 1991, *Zootermopsis*: Haverty et al. 1988). Despite the potential of chemo-taxonomy revealed by these studies, concerns for the method have still been raised. Distinct variations seen in the hydrocarbons within the spatial range of a single species imply a necessity for scrupulous interpretations of cuticular hydrocarbon patterns (Kaib et al. 2002).

Genetic analysis through molecular techniques may be, at the present time, the most efficient means of non-morphological termite identification. A majority of this work is achieved by examining the DNA sequences from mitochondrial DNA (mtDNA) cytochrome oxidase II (COII), and 16S ribosomal RNA (rRNA) genes. In order for these methods to be taxonomically-important, model sequences are obtained from specimens that accurately fit the classical description of their respective species. They then serve as the type sequences by which unidentified specimens are compared. The fact that the type sequence is classified based on the morphology of the specimen from which it was isolated is sometimes viewed as a caveat to this system. This may be true, but the real benefits of molecular techniques are seen during the subsequent analyses of unknown or morphologically analogous specimens. Multiple sequences can be impartially interpreted with confidence because the rate of amplification error is minimal. In the case of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses of mtDNA 16s rRNA genes, the possibility of an intra-species allele insertion/deletion within the amplified base pair region is generally less than 0.05% (Lin et al. 2002, Szalanski et al. 2003). This translates into an evaluation method far more objective than measuring body parts, and certainly more so than making judgments on the appearances of those body parts.

Phylogenetic analyses using sequenced portions of mtDNA COII genes have proven effective at revealing surprising relationships between termite species (Miura et al. 1998, Jenkins et al. 1999, Austin et al. 2002, 2004a). PCR-RFLP is another molecular procedure widely accepted as a tool for identifying species of vertebrate animals (Wolf et al. 1999) as well as insects including *Muscidifurax* wasps (Taylor and Szalanski 1999), *Anopheles gambiae* Giles complex (Fanello et al. 2002), and *Rhagoletis* species (Salazar et al. 2002). Since the first effective differentiation of *Reticulitermes* by PCR-RFLP (Szalanski et al. 2003), the technique has been applied in numerous evaluations of subterranean termite taxonomy (Austin et al. 2004b, c, d, 2005; Brown 2005). The PCR-RFLP method is a dependable and objective means of discerning *Reticulitermes* species. With proper application, it should significantly assist in the revision of which morphological features are truly capable of diagnostically separating the species of this genus.

Regardless of species, termites of the *Reticulitermes* genus share certain biological traits that allow them to damage a structure in an almost clandestine manner. The cryptic nature of subterranean termites keep them out-of-sight, and thus many homeowners are unaware of their presence. These termites spend the majority of their life below ground or in wood directly in contact with the soil. Shelter tubes, made of soil particles mixed with secretions from the termite's fontanelle, can be constructed to bridge gaps between the soil and above-ground wood (Kofoid 1934, Triplehorn and Johnson 2005). Although *Reticulitermes* can survive in wood having high moisture contents, they principally rely on the soil for their water needs (Potter 2004). Hydration is not the only benefit gained from living in the soil; it can also provide more stable ambient temperature ranges, reduce exposure to light, and reduce the risk of predation (Lee and Wood 1971).

Within the soil where they live, subterranean termites can find a diversity of their favored cellulose-based food sources. Most often available to them are living wood, dead/decomposing wood, other plant debris, roots, and fungi (Lee and Wood 1971, Potter 2004). Having a steady supply of plant material nearby is helpful, but it does little good if it cannot be converted into usable nutrients. To accomplish this, termites first feed on these materials to acquire necessary plant polysaccharides such as cellulose, hemicellulose, and lignocellulose. The lower termites, which include *Reticulitermes*, rely on anaerobic flagellates of the genera *Dinenympha, Holomastigotes, Pyrsonympha, Spirotrichonympha,* and *Trichonympha* located in their hindgut to carry out the metabolic reactions responsible for breaking down the carbohydrates to glucose (Belitz and Waller 1998, Cook and Gold 1999). The termites can however, take some credit for the digestive work. Slaytor (1992) noted that termites produced endogenous cellulases from their salivary glands to facilitate the initial digestion of the cellulose prior to it reaching the hindgut. In members of *R. speratus* (Kolbe), the salivary glands, foregut, and midgut all showed signs of endo-β-1,4-glucanase and β-glucosidase activity despite having little to no involvement with protozoa (Slaytor 1992). Regardless, the lower termites depend immensely on their mutualistic symbionts to help them maintain their status as some of the most efficient cellulose recyclers in the soil (Martin 1991, Ohkuma 2003).

Fungi rank among subterranean termites' most abundant competitors for cellulose-based resources. According to Bridge and Spooner (2001), the bias of current methods for detecting the abundance of fungi in soil could not yield true values unless several techniques were combined. They estimated the number of soil-borne fungi species might range well over 80,000. An estimate given by Gilbertson in 1980 suggested between 1,600 and 1,700 of North American fungal species were wood-rotting basidiomycetes. The extreme population density of these fungi in the soil, especially in forest ecosystems, allows them to out-compete all other

organisms, even termites, for the title of chief decomposers of cellulose and lignin (Alexopoulus et al. 1996).

When the high biodiversity of fungi is mixed together with flourishing subterranean termite colonies in one soil community, questions about co-existence naturally arise. Archeological evidence shows that termites and certain species of fungi have been co-habitating the soils since at least the Early and Late Cretaceous Periods, roughly 135 million years ago (Kullnig-Gradinger et al. 2002, Krishna and Grimaldi 2003). This has provided ample time for specific co-evolutionary adaptations to mature within the relationships between these groups. The past century has seen extensive research in this area, but scientists still do not fully understand the multifaceted relationship taking place between termites and fungi.

Nearly all classes of fungi are in some fashion associated with termites, but the main groups that have been isolated from them are basidiomycetes and saprophytic soil fungi (Hendee 1933, Waller et al. 1987, Zoberi and Grace 1990). Samples from different substrates, including infested wood, soil, shelter tubes, external/internal surfaces of termites, and termite frass, demonstrated only minor overlaps in the fungal species identified. The majority of the saprophytic fungi were identified as members of the genera *Absidia, Cunninghamella, Mucor, Penicillium,* or *Trichoderma*. An additional explanation for such findings could be that the bias existing in fungal detection methods did not allow for the growth of some fungi. Regardless, these surveys have shown that subterranean termites can be exposed to over 70 species of fungi during foraging and feeding.

Further experimentations have been preformed to identify species-specific termite/fungus interactions. This type of research gained significance after Esenther et al. (1961) discovered that the subterranean termites they tested exhibited a preference for wood colonized by, or treated with an extract of a brown-rot fungus, *Gloeophyllum trabeum* (Persoon: Fries)

Murrill (formerly *Lenzites trabea*). In later tests, *Reticulitermes* spp. were shown to aggregate and stay at feeding sites inoculated with *G. trabeum* (Amburgey and Smythe 1977, Amburgey 1979, Cornelius et al. 2002). Recent tests have also shown that tunneling by *Reticulitermes* spp. is positively influenced by extracts made from *G. trabeum* (Rust et al. 1996, Su 2005). These studies demonstrated that the termites directed their foraging efforts toward areas treated with the fungal extract. Other incidents where specific fungal species encouraged termite activity have been mentioned throughout the literature (Sands 1969, Amburgey 1979, Esenther and Beal 1979, Cornelius et al. 2002, 2004).

Certainly not all termite-fungal relations promote the interests of termites. Interspecific competition is expected to develop when trophic resources overlap (Giller 1996), as they do in the case of termites and fungi. White-rot fungi attack all cell wall constituents by depolymerizing the cellulose, hemicellulose, and lignin of their host plant. While doing this, they metabolize the glucose and other polysaccharides at a rate equal to the formation of these depolymerization products (Highley 1973, Blanchette 1991, Zabel and Morrell 1992, Nakasone 1993, Hammel 1997). This removes a large portion of the host plant's nutritional value leaving little resources behind for other competitive organisms such as termites to consume. Through time, termites may have evolved to associate white-rot fungi with food supplies that yield lower energy resources. Perhaps this is one reason why white-rot fungi are generally thought to deter termite feeding on wood. Research by Amburgey and Beal (1977) showed that wooden stakes colonized with the white-rot fungus *Ganoderma applanatum* (Persoon) experienced less attack by termites over a 14 month period than did wood not colonized by this fungus. Their results showed that out of the 48 stakes in the study, 36 stakes not colonized by *G. applanatum* either were "destroyed" or suffered "heavy penetration and feeding" by termites. The 12 stakes that were colonized by the white-rot fungus only had minimal signs of termite activity. Furthermore,

they noted similar findings by Kovoor (1964) and Schultze-Dewitz and Unger (1972) in which termites avoided wood decayed by other white-rot fungi.

The type of termite response produced by fungi is not absolute, even at the species level. Each strain of a fungal species has the potential to generate a different behavioral response from termites (Becker and Kerner-Gang 1964, Lund 1965). Amburgey and Smythe (1977) found that, even with *G. trabeum,* the level of attraction exhibited by the termites depended on the particular strain being used. This could have been the reason why Grace et al. (1992) saw siderophore extracts of *G. trabeum* deter termite feeding. Added factors that could also influence the nature of the termite's response are geographical location, climate, the species of wood the fungus is growing on, and even the termite species in question (Sands 1969, Esenther and Beal 1979, Cornelius et al. 2004).

Since the 1940s, the principal approach to subterranean termite control has centered on soil termiticides (Lewis 1997). Several varieties of registered soil termiticides are currently available on the market. These products use an active ingredient (a.i.) such as bifenthrin, cypermethrin, fenvalerate, fipronil, imidacloprid, or permethrin to either kill or repel termites (Su 2002). Issues regarding the safety of these, and virtually all insecticides, has led to a decline in the public's tolerance concerning heavy pesticide applications. The latest a.i. to be discontinued as a soil termiticide in the United States was chlorpyrifos in January 2006 (USEPA 2005). This has strengthened an expanding movement toward subterranean termite control techniques that have less environmental risk and lower chemical exposure to humans (Forschler and Jenkins 2000).

One subterranean termite management strategy that falls into this category is the use of a baiting system. Lenz and Evans (2002) describe how in 1921, G.F. Hall began using a bait matrix treated with arsenical solutions to control termites. The baiting technique was later

supported by C. A. Kofoid (1934) who stated concerning termites that "the concentration of the colony and the grooming habits of its constituent individuals facilitate the distribution and eating of poison dusts by its members." This is the principle from which the entire baiting system strategy is rooted. Lenz and Evans (2002) further mention that the concept of installing monitoring stations around infested structures originated in 1955. At that time, L.G.E. Kalshoven placed wood-filled bamboo cylinders in soil to track termite activity near buildings. Ideas developed through the research of Esenther and Gray (1968) and Beard (1974) have further contributed to termite management via baiting systems. These systems have experienced a gradual evolution over time in their designs, techniques, bait matrix materials, and active ingredients.

The development and marketing of termite baiting systems has expanded, in a secondary manner, the experimentation with termite-fungal associations. In nearly all systems, a cellulose matrix is placed in the soil to act as the initial termite bait. Generally, there are no precautionary measures taken to exclude other cellulose-feeding organisms, such as fungi, from accessing the bait. This often leads to a problem for the pest control operators (PCOs) who are monitoring these products in the field. During inspections, many PCOs encounter these cellulose matrices colonized by various fungi. They must decide whether the presence of fungi on baits would be detrimental to termite feeding. This situation continues today because the literature has not convincingly demonstrated fungi as having a constant, predictable influence on termites. Even if certain fungal species were recognizable in the laboratory, positive identification of these species in the field would remain impractical for a PCO lacking mycological training.

One alternative to this dilemma would be to prevent fungal growth from forming on the cellulose matrix while maintaining its attractancy to termites. A number of possible approaches exist for impeding the colonization of fungi on the bait. One method that naturally comes to

mind is the pre-treatment of the baits with a fungicide. With the necessity of maintaining the bait's palatability to termites and the concerns of applying chemicals to the soil, the option of using fungicides seems daunting. Perhaps a more unconventional tactic would be to inoculate the bait with a form of a biological control.

One interesting option is the application of mycoparasitic fungi, such as species of *Trichoderma* (Persoon: Fries). These free-living, saprophytic fungi have several ecological and biological characteristics that make them ideal for use as biocontrol agents in termite baiting systems. On average, 10^1-10^3 culturable propagules of *Trichoderma* can be found inhabiting a single gram of any given soil type (Harman et al. 2004). They can easily establish themselves in numerous ecological niches because of their aptitude for tolerating and adjusting to fluctuations in soil dynamics (Papavizas 1985). While inhabiting these niches, *Trichoderma* spp. proliferate rapidly, successfully competing against other fungi for nutrients (reviewed by Papavizas 1985, Schoeman et al. 1999, Howell 2003). Production of secondary metabolites, such as siderophores and antibiotics, allow *Trichoderma* spp. to invade and overpower weaker, susceptible fungi (Di Pietro et al. 1993, Baek et al. 1999, Khetan 2001, Vey et al. 2001).

One important metabolite produced by *Trichoderma* and several other genera of fungi is the epidithiodiketopiperazine antibiotic, gliotoxin (Benedict and Langlykke 1947, Vey et al. 2001). This secondary fungal metabolite is placed in the epipolythiodiozopiperazine (ETP) class of toxins because of its internal disulphide bridge (Fig. 1) (Waring and Beaver 1996, Gardiner et al. 2005). The antifungal properties of gliotoxin were first discovered through experimentations with *Gliocladium* and *Trichoderma* (Weindling and Emerson 1936, Weindling 1937). Research has revealed that these properties are derived from the sulphur bridge (Müllbacher et al. 1986) and can synergistically interact with fungal cell-wall degrading enzymes to enhance the biocontrol ability of *T. virens* (Miller, Giddens and Foster) von Arx (Di Petro et al. 1993).

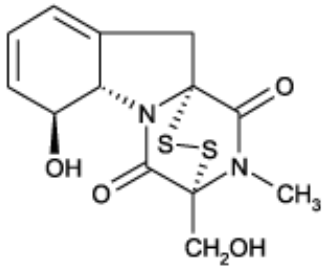

Fig. 1. Chemical structure of gliotoxin.

Due to these characteristics, a species of *Trichoderma* such as *T. virens* would seem a likely candidate to incorporate into the biocontrol of fungi in termite baiting systems. Reviews by Papavizas (1985), Schoeman et al. (1999), and Harman et al. (2004) have thoroughly covered the application of *T. virens* and other *Trichoderma* species in a variety of agricultural-related biocontrol situations. As a result, further information on *T. virens* covered here will only be in respect to its qualifications as a biological wood preservative.

Since the early 1950s, researchers have been testing the capacity of *Trichoderma* spp. to control decay fungi on wood and living trees (Lindgren and Harvey 1952, Hulme 1970, Hulme and Shields 1972, Mercer and Kirk 1984a, 1984b; Bruce and Highley 1991, Highley et al. 1994). Work by Highley et al. (1997) specifically focused on the importance of gliotoxin, produced by *T. virens*, as a biocontrol against wood decay fungi. Tests with gliotoxin-producing *T. virens* (GLT+) strains and gliotoxin-deficient (GLT-) mutants showed only slight differences in their abilities to control decay fungi. Wood blocks treated with a GLT+ strain experienced no measurable weight loss from any of the wood-rotting basidiomycetes tested. White-rot fungi did however cause, on average, weight loss between 13 and 63% in wood blocks treated with GLT- strains. Wood blocks vacuum-impregnated with filtrates of a GLT+ strain lost 4-14% and 15-50% of their weight due to brown-rot and white-rot fungi, respectively. These results showed

that gliotoxin, when applied as a stand-alone treatment, did not inhibit decay fungi as well as live fungal inoculations (Highley et al. 1997). *Trichoderma virens* therefore relies on a tactical combination of its characteristics to successfully parasitize pathogenic fungi in the soil (Highley et al. 1997, Howell 2003, 2006).

Recently, the holotype of *T. virens* was discovered, causing the fungus to be reclassified as *Hypocrea virens* Chaverri, Samuels and Stewart (Chaverrie et al. 2001). Research studies and biological control work continue to be conducted on *T. virens* because of the inability to produce *H. virens* in laboratory conditions. Experiments performed for this thesis used *T. virens*; therefore, the fungus is only referred to in its anamorph form and not as *H. virens*.

The purpose of the research presented in this thesis was to extend the scope of biocontrol of wood decay fungi by studying the effects of *T. virens* on non-target, subterranean termites. In order to properly accomplish this, experiments were performed with the following objectives: (1) to determine if extracts taken from strains of *T. virens* influence the feeding activity of *R. virginicus* [Null Hypothesis: *T. virens* extracts induce no significant difference in termite feeding] (Chapter II), (2) to determine if the presence of live *T. virens* on wood influences the feeding activity of *R. virginicus* [Null Hypothesis: having *T. virens* on wood makes no significant difference in the feeding activity of termites] (Chapter II), (3) to determine if strains of *T. virens* vacuum-impregnated into wood influence the feeding activity of *R. virginicus* [Null Hypothesis: vacuum impregnation of *T. virens* into wood creates no significant difference in termite feeding] (Chapter II), (4) to evaluate *Reticulitermes* spp. identification techniques that rely on morphometrics of soldier labra [Null Hypothesis: the identification technique does not reveal significant differences in labral size among *R. flavipes*, *R. hageni*, *R. tibialis*, and *R. virginicus*] (Chapter III).

The fourth objective addressed identification of *Reticulitermes* species. The initial identifications of field-collected termite samples were based on morphological characteristics. Sample identifications made following morphological descriptions of species (Scheffrahn and Su 1994, Hostettler et al. 1995) often contradicted one another when compared. These equivocal results led to the decision to use PCR-RFLP analysis of the mtDNA 16S rRNA gene, described by Szalanski et al. (2003), as a diagnostic alternative. By testing the null hypothesis, a conclusion was reached regarding the use of soldier labra measurements for identifying termite species. These results warranted inclusion into this thesis and therefore have been outlined separately in Chapter III.

The findings of this thesis will further promote advanced research into aspects of the subterranean termite biology, control, and taxonomy. These experiments help to provide insight into whether or not subterranean termite feeding is impacted by *T. virens*. Future research should be done to analyze the influence of this fungus on termite feeding in field situations. There is the potential for these results to lead to a new direction in termite baiting systems. Before truly efficient control can be accomplished however, an accurate identification of the pest must be made. The identification procedures shown in this thesis support continued investigation into the use of genetic analysis to clarify diagnostically-important morphological characteristics of termite species. As is the case with all research, the broader objective of this thesis was to expand the field of science by uncovering novel information, and perhaps to foster the ambitions of other individuals to test the unknown.

CHAPTER II

EFFECTS OF *Trichoderma virens* (HYPOCREALES: HYPOCREACEAE) ON THE FEEDING BEHAVIORS OF *Reticulitermes virginicus* (ISOPTERA: RHINOTERMITIDAE)

Introduction

There is a need for the development of new control strategies and the improvement of methods currently employed against subterranean termites. The principal means of termite control, since the 1940s, has been the creation of soil barriers through the application of liquid termiticides (Lewis 1997). PCOs have nearly exhausted the gamut of insecticides by utilizing termiticides with an a.i. from almost every chemical class (Su and Scheffrahn 1998, Su 2002, Potter 2004). Public health concerns over the persistence of these chemicals in the environment led to the discontinuance of many termiticide formulations (Su and Scheffrahn 1998). These feelings strengthened the public's desire for low environmental risk, low human exposure termite control practices (Forschler and Jenkins 2000).

Termite baiting systems have become popular alternatives to liquid soil termiticide treatments. This strategy involves the placement of bait stations at preset intervals and in areas of known or suspected termite activity (Potter 2004). Stations are typically installed below ground and filled with an untreated cellulose material, such as wood or cardboard, which encourages foraging termites to feed at the location. Once the termites become well established within a station, bait containing an a.i. is put in place of the untreated cellulose. Aside to controlling the termite problem, a major benefit of this process is a reduction in the chemical exposure experienced initially by the PCO, and later by the customer. This technique is also

suitable in environmentally delicate situations, making it an alternative solution to infestations occurring in close proximity to bodies of water. Baiting systems have become a legitimate termite management tool with several advantages, but that does not mean they are clear of all obstacles.

Overall baiting effectiveness hinges on the biology of the subterranean termite. Foraging patterns of *Reticulitermes* spp. fluctuate in regard to seasonal changes in weather and colony composition (Houseman 1999, Glenn 2005). These factors seem to influence when bait stations are most likely to be discovered and fed upon by the termites. In many cases, subterranean termites prefer consuming wood that is in the process of being decayed by fungi (Hendee 1933, Esenther et al. 1961, Smythe et al. 1971, Cornelius et al. 2004). During foraging and feeding, subterranean termites are exposed on average to over 70 species of fungi (Hendee 1933, Waller et al. 1987, Zoberi and Grace 1990). However, termites do not respond identically to all fungi. Some species of wood-rot fungi are known to inhibit termite feeding, repel foragers, or ellicit no response at all (Becker and Kerner-Gang 1964, Lund 1965, Amburgey and Beal 1977, Grace et al. 1992). This creates a problem in the termite baiting system because the cellulose materials used to entice the termites are susceptible to fungal decay. If fungi colonize these materials, identifying the species in the field would be extremely difficult, if not impossible in some cases. This means an accurate determination of how the fungus, or fungi, will affect termite behavior cannot be made.

Research efforts have not provided a conclusive answer as to which fungi are consistently beneficial, detrimental, or neutral to termite behavior. Testing the relationship between *Reticulitermes* spp. and all the fungi they may encounter is not realistic. Far too many variables are capable of altering the outcome of termite-fungal interactions. Examples of these variables include climate, the material the fungus is growing on, the length of time the material is

colonized by the fungus, the strain of the fungus, and even the termite species (Lund 1965, Sands 1969, Amburgey and Smythe 1977, Esenther and Beal 1979, Cornelius et al. 2002, 2004). One simple solution could be to replace the decaying materials, but this would be labor-intensive, cause unnecessary disturbance within the bait stations, and possibly waste effective bait. Another option is to prevent the growth of decay fungi on the cellulose materials used in the bait stations.

A biocontrol approach to abate fungal growth may be the most suitable tactic in a termite baiting system. The saprophytic fungus *T. virens*, commonly found in soils throughout the world, is regularly applied in agricultural situations as a biocontrol agent against pathogenic fungi (Papavizas 1985, Schoeman et al. 1999, Harman et al. 2004). The ability of this fungus to proliferate rapidly and produce toxic secondary metabolites allows it to sequester more nutrients by repelling or out-competing other fungi (Papavizas 1985, Di Petro et al. 1993, Baek et al. 1999, Khetan 2001, Vey et al. 2001, Harman et al. 2004). One secondary metabolite produced abundantly by 'Q' strains of *T. virens* is gliotoxin, an epidithiodiketopiperazine antibiotic (Benedict and Langlykke 1947, Howell et al. 1993, Vey et al. 2001). Under laboratory conditions, strains of *T. virens* or solutions of gliotoxin were both able to protect wood against brown rot and white rot fungi (Hulme 1970, Hulme and Shields 1972, Highley 1997, Highley et al. 1997). At the very least, this shows the potential, *T. virens* has for use in termite bait stations; however, no research has examined the reaction of *Reticulitermes* spp. to wood inoculated with this fungus.

The objectives of these experimentations were to examine the feeding behavior of *R. virginicus* when presented food inoculated with *T. virens*. Feeding assays were performed using two strains of *T. virens* tested in three forms: liquid extracts, live mycelia, and homogenated mycelia.

Materials and Methods

Termite Collection. Populations of *R. virginicus* were collected from Emerald Forest Park located in College Station, Texas, between the months of May and July 2005. This park was chosen based on its accessibility, low degree of human disturbance, and conditions conducive to termite activity. A collection site bordering mature *Quercus* spp. (Oak) and *Ilex vomitoria* (Yaupon Holly) was selected inside the park. In-ground bucket-bait traps, similar to those used by Su and Scheffrahn (1986), were installed in areas where subterranean termite activity was found. Traps were placed in the ground by digging a hole approximately 20 cm in diameter and 25 cm deep. A small plastic bucket, having the bottom half removed, was inserted into the hole so that the top edge was flush with the ground surface. A piece of aged pine (2 x 9 x 12 cm) was positioned in the base of the trap and on top of which, further bait was placed. Two bait types were used in the bucket-bait trap systems. The first bait type consisted of a wooden sandwich (Fig. 2A) comprised of four 2 x 9 x 12 cm aged pine pieces bolted together with a single bolt and wing nut. The second bait type consisted of 40-45 strips of corrugated cardboard (0.3 x 4 x 12 cm) packed into a polyvinyl-chloride (PVC) pipe (13 cm height, 10 cm inside diameter) (Fig. 2B,C). Cardboard strips were held in place by wire mesh (0.6 x 0.6 cm openings) affixed to one end of the PVC pipe. A single PVC pipe filled with cardboard was placed on top of a 2 x 9 x 12 cm piece of aged pine in the base of the trap. Wooden sandwiches were used as the initial bait in each trap and remained there until consistent termite feeding was established inside the trap. Once this occurred, the wooden sandwich was removed and replaced with cardboard bait.

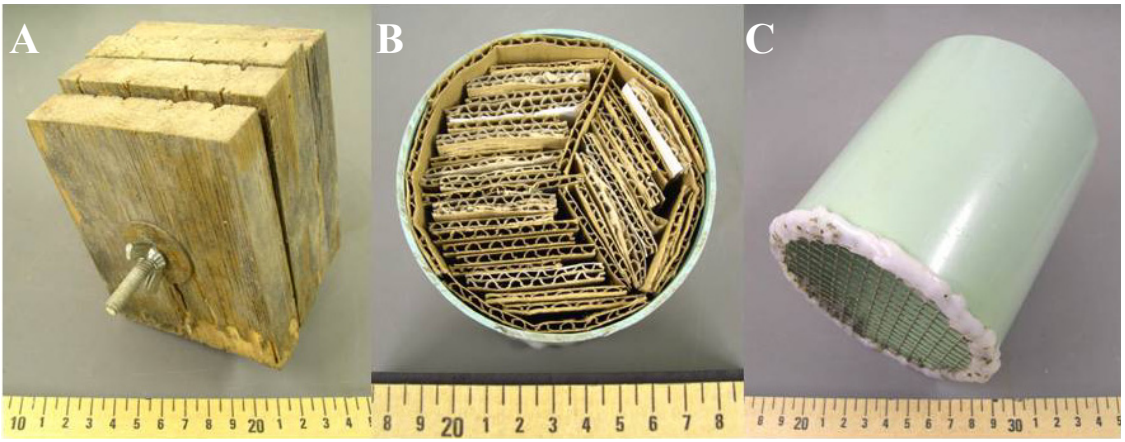

Fig. 2. Bait types used in bucket-bait traps to collect subterranean termites, (A) wooden sandwich, (B) PVC pipe filled with cardboard (top view), (C) PVC pipe (side view).

All baits having termite activity were collected on a weekly basis and returned to the laboratory where they could be properly disassembled. The termites were physically removed from the bait pieces and further separated from any loose debris using sorting trays (Fig. 3). Soldiers were frequently removed at random and identified to species using the labral morphology techniques described in detail in Chapter III and with the methods of Hostettler et al. 1995. The termite identifications were consistently *R. virginicus*, which PCR-RFLP analysis later confirmed (Chapter III). The collected termite colonies were maintained in plastic petri dishes and fed moistened tongue depressors until their use in experiments (Fig. 4). Petri dishes containing termite colonies were housed in plastic shoeboxes (30 x 15 x 9 cm) that had a thin layer of wetted sand in the bottom. A sheet of aluminum foil was placed over the sand to help maintain proper moisture and humidity levels inside the shoeboxes. Termite colonies were reared at temperatures ranging from 24-30°C and light-dark settings following an approximate 12 h cycle.

Fig. 3. Sorting tray and collection box used for separating termites from trap debris.

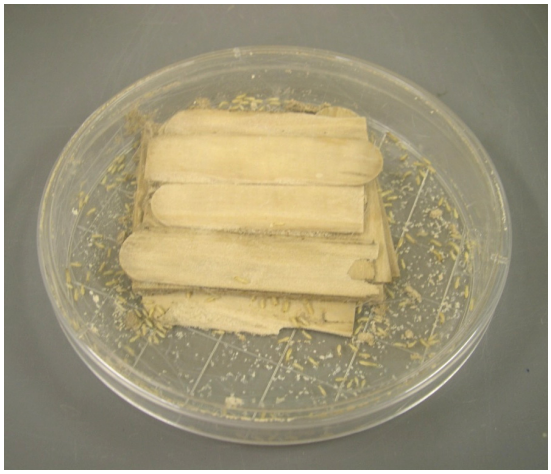

Fig. 4. Petri dish with moistened tongue depressors serving as artificial nest and food source for collected termites.

Fungal Strains. Strains 'P' and 'Q' of *T. virens* were cultured for use in these experiments. The gliotoxin-deficient form (GLT-) of *T. virens* chosen was Tv11c165, a representative of the 'P' strain. The 'Q' strain, Tv29-8, served as the gliotoxin-producing isolate (GLT+) of *T. virens*. Conidia of each strain were obtained from the -80ºC stock cultures at the Department of Plant Pathology and Microbiology, Texas A&M University (College Station,

TX). These samples were plated onto potato dextrose agar (PDA) (Sigma-Aldrich Co., St. Louis, MO) in 100 x 15 mm petri dishes, and held at 28±2ºC for five days. These parent cultures were allowed to grow until the medium was fully colonized.

A conidial concentration of each parent culture was determined using a hemocytometer. To accomplish this, 1.0 ml of distilled water was applied to the culture surface and gently spread into an approximate 2.5 cm diameter circle. The mixture of conidia and water was pipetted and used to make a 1:100 dilution. From the 1:100 dilutions, spore counts were conducted using the hemocytometer. A 2.5 ml sample from each of the 1:100 dilutions was used to inoculate the respective culture media designated in the following bioassays.

Fungal Extract Bioassay. The first no-choice bioassay tested the feeding behavior of *R. virginicus* toward filter paper disks inoculated with culture filtrates of the strains. This test was designed to determine if the secondary metabolites produced by *T. virens* would effect termite feeding when applied directly to a food source. The GLT+ and GLT- strains were grown in 100 ml of liquid Weindling's medium (per 1 liter water: 25 g glucose, 5 g phenylalanine, 2 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$) for 15 d. As a control, 100 ml of uninoculated liquid Weindling's medium was used. These cultures were swirled at 90 rpm using The Belly Dancer® (Model US BDbo, Stovall Life Science Inc., Greensboro, NC) while in an environment of 28±2ºC with diurnal alternation of light and dark cycles.

The termites used in this bioassay were collected from Emerald Forest Park site 1 on 27 May 2005. Termites were starved for 9 h preceding their use in the experiment. A total of 75 worker termites and three soldiers were placed in 100 x 15 mm petri dishes for each repetition in the feeding series. A feeding series was defined as the six repetitions of each treatment that included termites. Non-feeding series were then defined as the six repetitions of each treatment

without termites. The four treatments included: (1) distilled water control (W), (2) blank media control (M), (3) GLT- strain (P), and (4) GLT+ strain (Q).

After 48 h of culture growth, a 4 ml sample of each treatment was drawn and then passed through a 25 mm, 0.45 µm mixed cellulose esters (MCE) sterile syringe filter (Fisherbrand®, Fisher Scientific International Inc., Hampton, NH) to remove any large particles, such as mycelial matter in the case of fungal treatments. A 12.5 µl amount of the filtrate was then applied to a 7.15 mm diameter disk cut from #3 qualitative circles, 70 mm filter paper (Whatman International Ltd, Maidstone, UK). A filtrate-treated disk was placed in each repetition of the respective treatment's feeding and non-feeding series. These procedures were carried out for each of the four treatment types every 24 h during the next 6 d, and then again at day 15 of culture growth. To maintain moisture levels, 10 µl of distilled water was applied at 36 h of testing and again at 60 h. Every new set of treatments represented a different fungal incubation period and a separate trial; this resulted in seven trials overall. No disk received a second application of filtrate. After 72 h of testing, repetitions for each treatment were concluded and data were collected.

Live Mycelia Bioassay. The second no-choice test involved offering wood inoculated with the live mycelia of *T. virens* to *R. virginicus*. Two types of wood were utilized during this experiment including southern yellow pine (SYP) (Idaho Timber Corp., Fort Worth, TX) that had been exposed to natural weather conditions for over six months, and Sentricon® Termite Colony Elimination Systems monitoring devices (SMD) (Dow AgroSciences LLC, Indianapolis, IN). The wood was sliced into pieces 1.2±0.2 mm thick using a compound miter saw (Model 36-220 Type 3, Delta International Machinery Corp., Pittsburg, PA) fitted with a fine finish blade (Robert Bosch Tool Company, Mount Prospect, IL). Disks were then cut to their final size of 1.2±0.2 mm thick and 9.5 mm in diameter with a #6 cork borer. In order to rid disks of excess

moisture, they were dried for 24 h in a Fisher Scientific isotemp oven (Model 625G, Fisher Scientific International Inc., Hampton, NH) set at 55±5ºC. All disks were then weighed and digitally photographed using the camera system described later in the Data Analysis section.

The GLT+ and GLT- *T. virens* strains were cultured on PDA for this experiment. One day after inoculating the medium, wood disks were placed in a circle on the PDA surface. Wood disks arranged in the same manner on uninoculated PDA served as blank medium controls. The disks remained in the cultures for a 7 d period allowing mycelia to completely cover their surface. All disks were removed from their respective cultures and allocated for use in either feeding or non-feeding series. A feeding series was defined as the six repetitions of each treatment that included termites. Non-feeding series were then defined as the six repetitions of each treatment without termites. Both series included the four treatments, W, M, P, and Q.

The termites for this bioassay were collected from Emerald Forest Park site 1 on 8 July 2005. An average weight of 1.97 mg per termite was used to estimate the 300 workers required per repetition. This weight was based on the mean weight of five sets of 25 termites, which equaled 49.2 mg. An additional 10 soldiers were added per feeding series repetition. The termites were placed in 100 x 15 mm petri dishes and subjected to a 12 h starvation period before being given a treated SYP or SMD disk. Disks in treatment W received 12 µl of distilled water daily to maintain moisture levels comparable to those of the other treatments. All repetitions were stopped in the feeding and non-feeding series after 48 h, because consumption on some disks had reached 75% or greater. After removal, disks were oven dried at 55±5ºC for 24 h, then weighed and digitally photographed for data collection.

Vacuum Impregnation Bioassay. During the third no-choice test, the feeding preferences of *R. virginicus* toward SYP and SMD disks (1.2±0.2 mm thick, 9.5 mm diameter) vacuum-impregnated with homogenated *T. virens* mycelia was examined. GLT+ and GLT- *T.*

virens strains were cultured in 100 ml of liquid Weindling's medium. An additional 100 ml of uninoculated medium was used as a control. Cultures were swirled at 90 rpm on The Belly Dancer® and maintained for 7 d in an environment of 28±2ºC with diurnal alternation of light and dark cycles. On the seventh day, 70 ml of the GLT+ culture was mixed with 280 ml of distilled water and then blended for 10 s using a BlendMaster® blender (Model 50200, Procter-Silex Inc., Southern Pines, NC). This was repeated with the GLT- culture, blank medium control, and distilled water control. These homogenated mixtures were added to their respective vacuum chambers containing SYP and SMD disks. In order to keep the wood disks submerged, they were held between two pieces of wire mesh (0.6 x 0.6 cm openings). The wood disks had been previously oven dried at 55±5ºC for 24 h, weighed, and digitally photographed prior to their placement in the chambers.

A specially-designed vacuum system (Fig.5) was created to impregnate the wood disks with homogenate. This system consisted of four separate vacuum chambers linked to a vacuum/pressure diaphragm pump (Model L-79200-00, Cole-Parmer Instrument Company, Vernon Hills, IL). Plastic containers (1.6 liter) (Heritage Mint Ltd., Scottsdale, AZ) served as the vacuum chambers, while a series of SAE J844 type B plastic tubing (Weatherhead® NT10008, Eaton Corp., Eden Prairie, MN) and brass fittings connected them to the pump. In order to impregnate the wooden disks thoroughly, the vacuum was maintained at 505±5 mmHg for 10 h. The disks were removed and allowed to air dry for 6 h before they were used in feeding and non-feeding series.

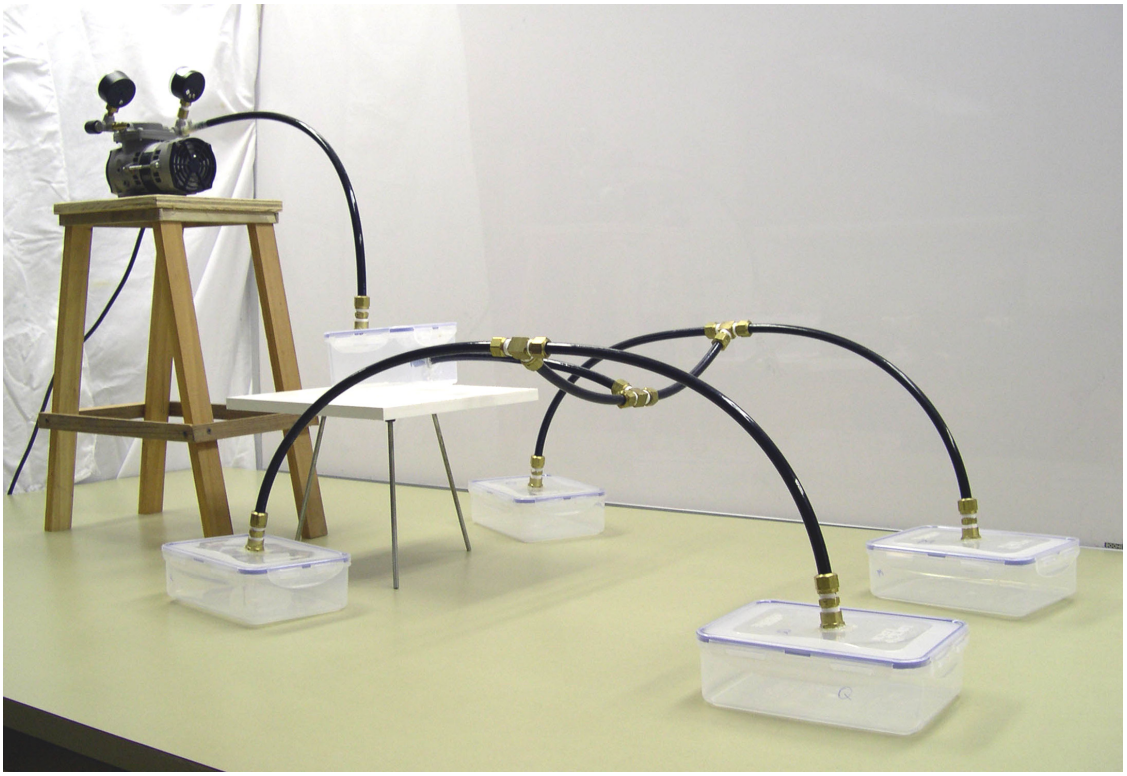

Fig. 5. Vacuum system used to impregnate wood disks with homogenated treatments.

The experimental design was similar to the one used during the Live Mycelia Bioassay. Collection of the termites occurred on the 10th and 17th of June 2005 from Emerald Forest Park site 1. Approximately 300 worker and 10 soldier termites were used in each repetition of the feeding series. The number of worker termites was estimated based on a calculated weight of 2.14 mg per termite. This was determined from the mean weight of five sets of 25 termites (53.6 mg) from the colony. All termites were removed from food for 12 h preceding the initiation of the experiment. Individual treated disks were placed into 100 x 15 mm petri dishes with termites (feeding series) or without termites (non-feeding series). A feeding series was defined as the six repetitions of each treatment that included termites. Non-feeding series were then defined as the six repetitions of each treatment without termites. Disks were re-hydrated with 12 µl of distilled

water at 36 h and again at 60 h after their placement in the petri dishes. The bioassay was stopped after 72 h, disks were oven dried at 55±5ºC for 24 h, weighed, and digitally photographed.

The efficacy of the *T. virens* mycelia was tested after completing the 10 h vacuum cycle. A 1 ml sample of the homogenated blend from each vacuum chamber was used to inoculate PDA. In addition, vacuum-impregnated disks were placed on PDA to check for efficacy of inoculation. These cultures were maintained at 28±2ºC with diurnal alternation of light and dark cycles. The viability of the homogenated *T. virens* mycelia and the presence of the fungus on/in the disks was determined by monitoring culture growth daily.

Data Analysis. The food disks used in all three bioassays were measured in terms of percent area lost. Consumption of wood disks were also measured in terms of weight loss. Area loss measurements were accomplished by analyzing digital images taken of each disk before and after testing, similar to the analyses done by Su and Messenger (2000) and Vahabzadeh (2002). In regard to wood disks, photographs were taken after the disks had been oven dried at 55±5ºC for 24 h. Disks were photographed from a distance of 3 cm using a digital camera (Nikon Coolpix® 4300, Tokyo, Japan) set on the macro close-up mode with no flash. The camera was mounted onto a copy stand (No. CS-3, Testrite Instrument Co. Inc., Newark, NJ) for stability (Fig. 6). All images were downloaded and opened under Adobe Photoshop (Adobe Systems Inc. 2001) where they were converted to a black-and-white color scheme to increase measurement accuracy. This required translating the images' color to grayscale and setting the contrast to a maximum level. A paint tool was used to correct small blemishes that remained inside the perimeter of the food disk after the color conversion. The amended images were analyzed using SigmaScan® Pro 5 (SPSS Inc. 1999) to determine the area of each disk. Calculations were then performed to determine the percent area loss for all disks.

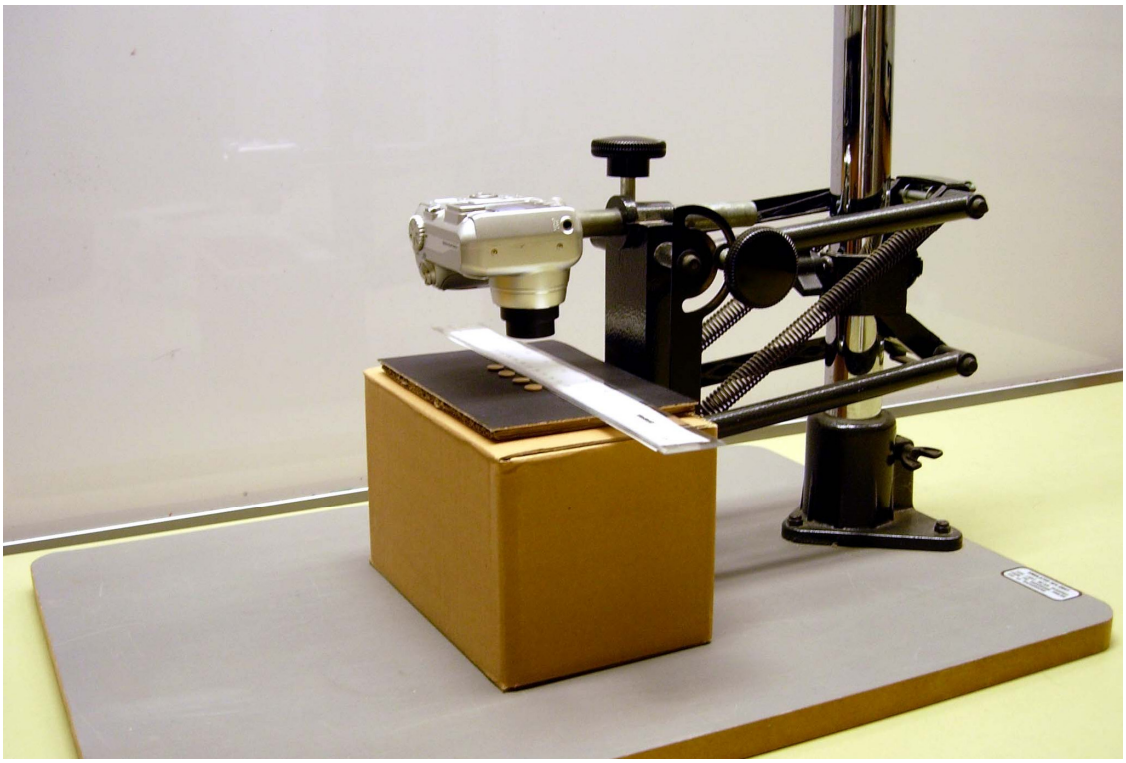

Fig. 6. Camera system for photography of filter paper and wood disks. Distance between camera lens and disks equaled 3 cm.

Percent weight loss was also measured of wooden disks involved in the Live Mycelia and Vacuum Impregnation Bioassays. This was accomplished by calculating the differences in weight between oven dried disks pre- and post-feeding. In all situations, extraneous variables, such as changes induced by the fungus, were corrected for by applying a treatment's mean loss during the non-feeding series to its respective data from the feeding series. For example, if disks in treatment W had a mean weight loss of 1.5 mg in the non-feeding series, then 1.5 mg was added to the final weight of each disk in the that treatment.

Data were analyzed for statistically-significant differences using SPSS® 14.0 (SPSS Inc. 2005). Univariate analysis of variance (ANOVA) ($\alpha = 0.05$) was run with the percent loss means in all bioassays to determine if statistically-significant differences existed. When groups

were shown to have significant differences, Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$) was performed to identify the differences. To assess the accuracy of area measurements derived from the digital images, bivariate correlations were run between percentages in weight loss and area loss for the Live Mycelia and Vacuum Impregnation Bioassays. Pearson's correlation coefficients were determined using a two-tailed significance level ($\alpha = 0.01$). Linear *R* squared values were acquired from the best fit line of the scatter plot.

Results

Fungal Extract Bioassay. During this no-choice test, the effects that *T. virens* strains had on filter paper consumption by *R. virginicus* varied depending on the age of the fungus culture. Disk consumption, in terms of the mean percent area loss for each treatment through all seven trials, is represented in Fig. 7. The blank medium culture was utilized in another experiment before the 15 d period was completed; therefore, no data were available for its Day 15 trial. Statistically-significant differences ($\alpha = 0.05$) were observed among the control and fungal treatments during all of the trials except Day 5 ($F = 0.843$; df = 3, 24; $P = 0.487$) and Day 15 ($F = 0.716$; df = 3, 24; $P = 0.505$) (Table 1). During the Day 2 trial, filter paper disks treated with the GLT- strain suffered significantly more area loss than the remaining treatments ($F = 7.64$; df = 3, 24; $P < 0.05$). The only significant difference occurring in the Day 3 trial was between the blank media control and GLT+ treatments ($F = 3.04$; df = 3, 24; $P < 0.05$). Disks in the Day 4 trial that were inoculated with *T. virens* differed significantly between strains ($F = 4.58$; df = 3, 24; $P < 0.05$), but not from either control treatments. The GLT+ treated filter paper in the Day 6 trial had significantly more area loss than all other treatments ($F = 5.23$; df = 3, 24; $P < 0.05$). During the Day 7 trial, the GLT+ extract was significantly different from all other treatments ($F = 28.5$; df = 3, 24; $P < 0.05$).

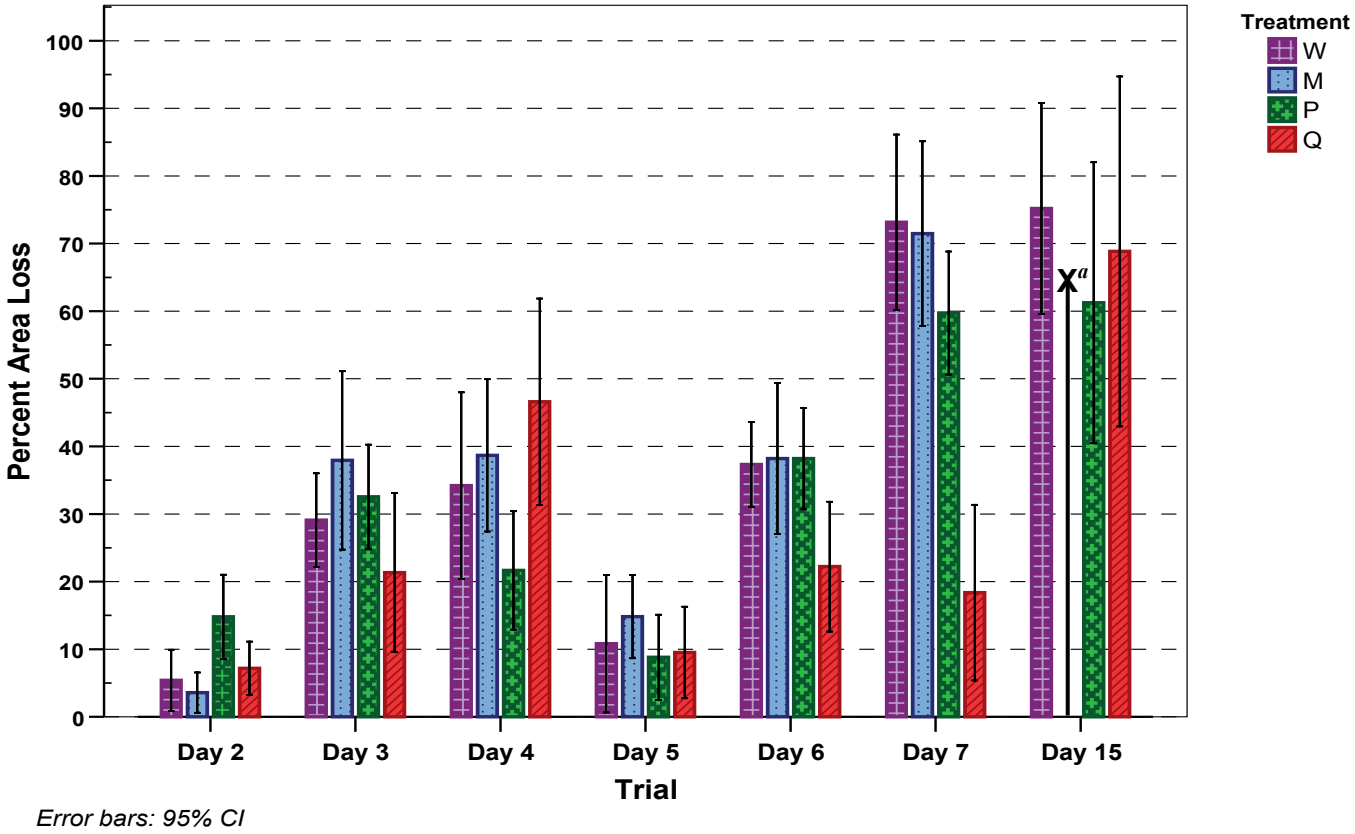

Fig. 7. Mean area loss of filter paper disks due to *R. virginicus* feeding per fungal incubation period during the Fungal Extract Bioassay. Trial days indicate age of cultures when treatment samples were taken. M, blank medium control; P, GLT- strain extract; Q, GLT+ strain extract; W, distilled water control.

[a] Blank medium culture was no longer available due to its use in another experiment.

Table 1. Mean (± SD) area loss of filter paper disks per fungal incubation period due to *R. virginicus* feeding during the Fungal Extract Bioassay.

Treatment[a]	Percent area loss per fungal incubation period[b]						
	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 15
W	5.41 ± 4.31a	29.10 ± 6.60ab	34.20 ± 13.15ab	10.84 ± 9.70a	37.34 ± 5.96a	73.15 ± 12.38a	75.19 ± 14.87a
M	3.58 ± 2.84a	37.94 ± 12.57b	38.68 ± 10.73ab	14.83 ± 5.87a	38.22 ± 10.64a	71.48 ± 13.02a	——
P	14.79 ± 5.95b	32.55 ± 7.35ab	21.66 ± 8.35a	8.81 ± 6.01a	38.22 ± 7.15a	59.73 ± 8.67a	61.27 ± 19.81a
Q	7.18 ± 3.76a	21.35 ± 11.20a	46.60 ± 14.55b	9.52 ± 6.44a	22.23 ± 9.16b	18.37 ± 12.38b	68.85 ± 24.67a
F value	7.64	3.04	4.58	0.843	5.23	28.5	0.716

Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$; df = 3, 24).
——, Blank medium culture was no longer available due to its use in another experiment.
[a] M, blank media control; P, GLT- strain extract; Q, GLT+ strain extract; W, distilled water control.
[b] Days indicate age of cultures when treatment samples were taken.

Filter paper disk area loss was also analyzed for statistically-significant differences ($\alpha = 0.05$) among the trails of individual treatments. Means tended to vary between trials, and as many as four homogenous subsets were seen in some treatments (Table 2). The distilled water control had four significantly different subsets ($F = 42.8$; df = 6, 42; $P < 0.05$), as did the GLT- extract treatment ($F = 25.3$; df = 6, 42; $P < 0.05$). Statistical variation within the blank medium control treatment was held to three subsets ($F = 33.1$; df = 6, 42; $P < 0.05$). The final treatment, the GLT+ extract, only revealed two homogenous subsets ($F = 16.7$; df = 6, 42; $P < 0.05$).

Table 2. Mean (± SD) percent area loss of filter paper disks per treatment due to *R. virginicus* feeding during the Fungal Extract Bioassay.

Trial	Percent area loss per treatment[a]			
	W	M	P	Q
Day 2	5.41 ± 4.31a	3.58 ± 2.84a	14.79 ± 5.95ab	7.18 ± 3.76a
Day 3	29.10 ± 6.60bc	37.94 ± 12.57b	32.55 ± 7.35bc	21.35 ± 11.20a
Day 4	34.20 ± 13.15c	38.68 ± 10.73b	21.66 ± 8.35abc	46.60 ± 14.55b
Day 5	10.84 ± 9.70ab	14.83 ± 5.87a	8.81 ± 6.01a	9.52 ± 6.44a
Day 6	37.34 ± 5.96c	38.22 ± 10.64b	38.22 ± 7.15c	22.23 ± 9.16a
Day 7	73.15 ± 12.38d	71.48 ± 13.02c	59.73 ± 8.67d	18.37 ± 12.38a
Day 15	75.19 ± 14.87d	———	61.27 ± 19.81d	68.85 ± 24.67b
F value	42.8	33.1	25.3	16.7

Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$; df = 6, 24).
———, Blank medium culture was no longer available due to its use in another experiment.
[a] W, distilled water control; M, blank media control; P, GLT- strain extract; Q, GLT+ strain extract.

Live Mycelia Bioassay. The wood disks used in the Live Mycelia Bioassay were measured for weight and area loss. The highest weight loss among SYP disks occurred in those treated with blank medium (67.78%), while the lowest were those covered with the mycelia of the GLT- strain (1.24%) (Fig. 8A). The same pattern existed in area loss for these disks (Fig.

8B). SMD disks treated with distilled water lost the most weight (55.88%), while those subjected to the GLT+ strains showed the least loss (14.01%) (Fig. 9A). Area loss in SMD disks followed the same trend as weight loss (Fig. 9).

The strength of correlation in the weight and area loss for SYP and SMD disks was analyzed. The level of correlation between weight and area loss in all treatments is shown for each wood type in Table 3. Weight and area loss among the SYP disks had a Pearson's correlation coefficient of 0.982 ($\alpha = 0.01$). The Pearson's correlation coefficient for weight and area loss in SMD disks was 0.970 ($\alpha = 0.01$). Scatter plots of the SYP and SMD weight and area loss data illustrates the correlation (Fig. 10). The best fit lines of these scatter plots revealed linear *R* squared values of 0.965 and 0.941 for the SYP and SMD data, respectively.

The *T. virens* treatments significantly decreased ($\alpha = 0.05$) consumption of SYP and SMD disks by *R. virginicus* (Tables 4 and 5, respectively). Thick mycelial mats covered the tops of disks preventing the termites from directly accessing the wood in those areas. As a result, termites were observed feeding on the underside of disks and in areas where mycelia were not present (Fig. 11). SYP disks covered with the mycelia of GLT+ and GLT- strains lost significantly less area than either the distilled water or blank medium treatments ($F = 33.55$; df = 3, 24; $P < 0.05$). In regard to the same disks, those treated with blank medium experienced significantly more area loss than did any other disks. Area loss among SMD disks treated with distilled water or blank medium was significantly greater than disks colonized by either *T. virens* strain ($F = 42.53$; df = 3, 24; $P < 0.05$). However, no significant variations were seen between the GLT+ and GLT- treated SMD disks.

The data gathered from the Live Mycelia Bioassay were analyzed for differences occurring between wood types in each treatment. Statistically-significant differences ($\alpha = 0.05$) were seen concerning the weight and area loss in the two wood types (Fig. 12). Within each

treatment, no significant difference was seen between the SYP and SMD disks based on area loss ($F = 36.00$; df = 7, 48; $P < 0.05$) or weight loss ($F = 30.56$; df = 7, 48; $P < 0.05$).

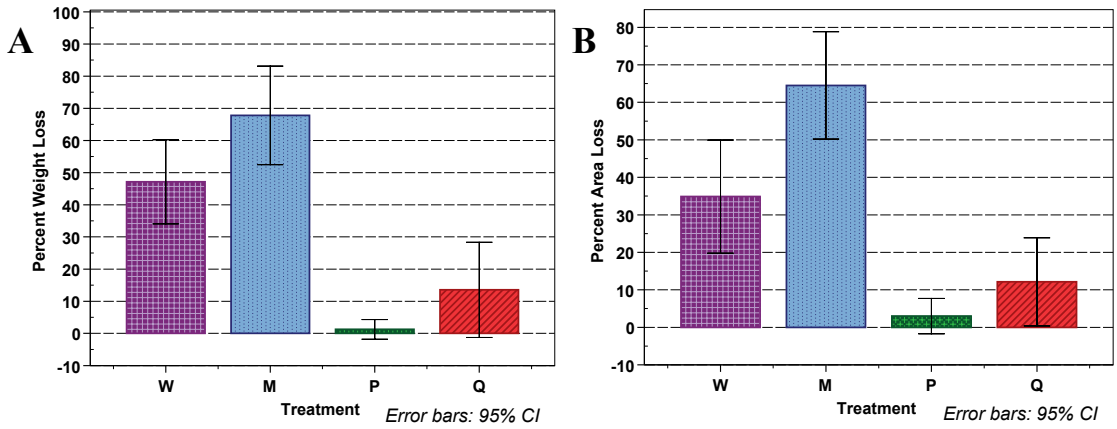

Fig. 8. (A) Mean percent weight loss and (B) mean percent area loss in southern yellow pine disks by *R. virginicus* during the Live Mycelia Bioassay. M, blank medium control; P, GLT- strain; Q, GLT+ strain; W, distilled water control.

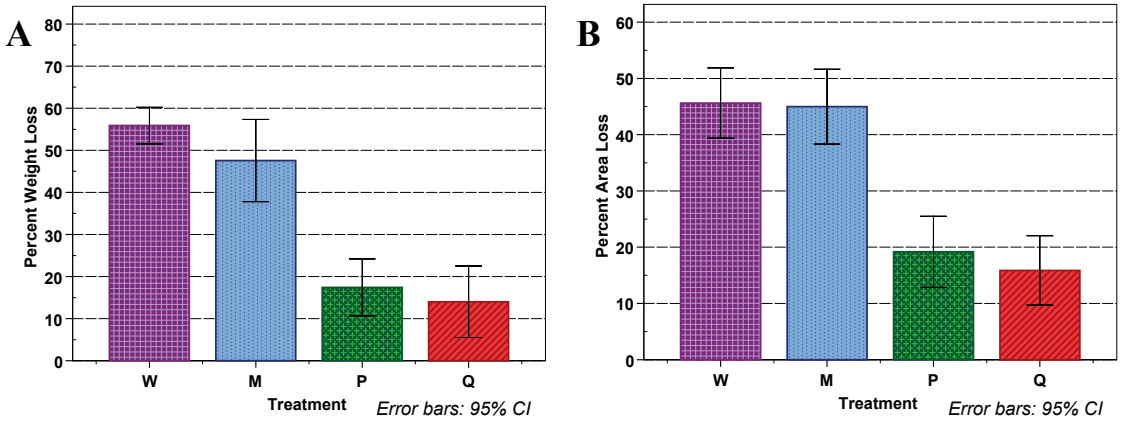

Fig. 9. (A) Mean percent weight loss and (B) mean percent area loss in Sentricon® monitoring device disks by *R. virginicus* during the Live Mycelia Bioassay. M, blank medium control; P, GLT- strain; Q, GLT+ strain; W, distilled water control.

Table 3. Correlation between weight and area loss in wood disks, regardless of treatment, during the Live Mycelia Bioassay.

		SYP[a]	SMD[b]
Mean ± SD	Weight	32.43 ± 29.23	33.72 ± 19.87
	Area	28.62 ± 26.56	31.42 ± 15.31
Pearson correlation		0.982**	0.970**

**indicates the correlation is significant at α = 0.01 (2-tailed).
[a] SYP, southern yellow pine disks.
[b] SMD, Sentricon® monitoring device disks.

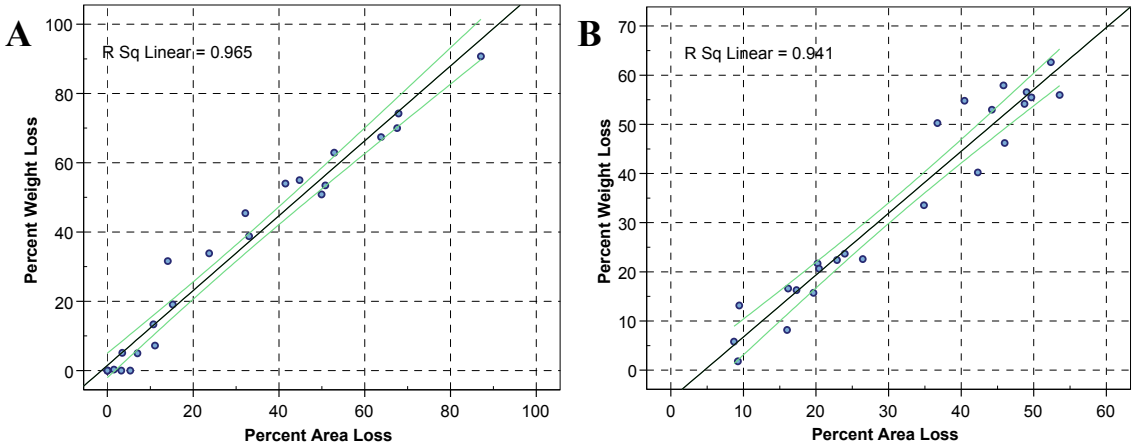

Fig. 10. Scatter plots representing the correlation between weight and area loss of (A) southern yellow pine and (B) Sentricon® monitoring device disks, respectively, used during the Live Mycelia Bioassay.

Table 4. Mean (± SD) percent loss in weight and area of southern yellow pine disks due to *R. virginicus* feeding during the Live Mycelia Bioassay.

Treatment[a]	Percent loss	
	Weight	Area
W	47.13 ± 12.48a	34.87 ± 14.36a
M	67.78 ± 14.59b	64.52 ± 13.64b
P	1.24 ± 2.92c	2.99 ± 4.47c
Q	13.55 ± 14.11c	12.12 ± 11.21c
F value	38.79	33.55

Means within a column followed by the same letter are not significantly different (α = 0.05; df = 3, 24).
[a] M, blank media control; P, GLT- strain extract; Q, GLT+ strain extract; W, distilled water control.

Table 5. Mean (± SD) percent loss in weight and area of Sentricon® monitoring device disks due to *R. virginicus* feeding during the Live Mycelia Bioassay.

Treatment[a]	Percent loss	
	Weight	Area
W	55.88 ± 4.15a	45.62 ± 5.95a
M	47.58 ± 9.31a	44.99 ± 6.34a
P	17.41 ± 6.46b	19.20 ± 6.02b
Q	14.01 ± 8.10b	15.87 ± 5.86b
F value	50.65	42.53

Means within a column followed by the same letter are not significantly different (α = 0.05; df = 3, 24).
[a] M, blank media control; P, GLT- strain extract; Q, GLT+ strain extract; W, distilled water control.

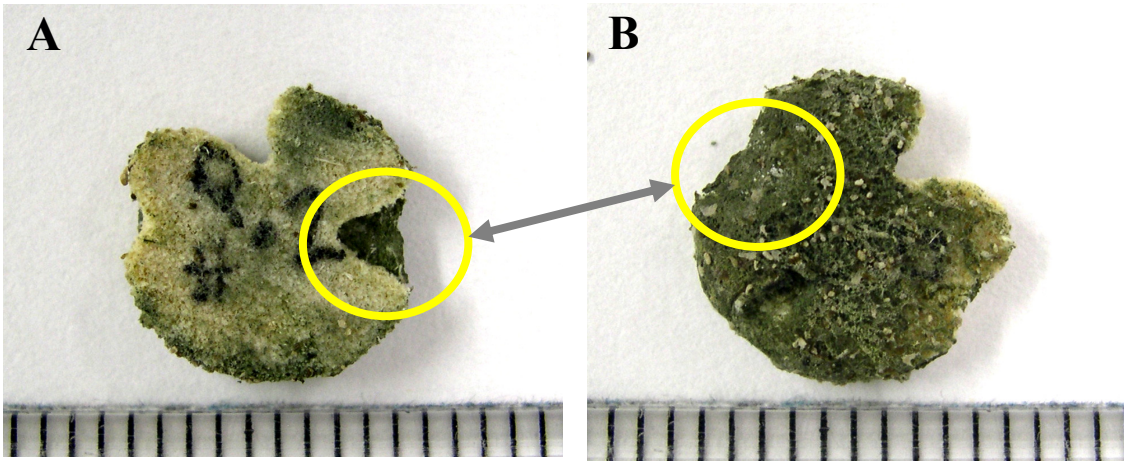

Fig. 11. Feeding damage to a Sentricon® monitoring device disk treated with GLT- strain during the Live Mycelia Bioassay. Circles indicate corresponding area of feeding on the disk's (A) underside and (B) topside covered with mycelia. The underside of the disk was in contact with the PDA during the 7 d growth period. This side was also placed downward on the surface of the petri dish during the feeding assay. Notice how the mycelia inside the circled area were not consumed once the termites worked their way underneath to the wood. Distance between black lines equals 0.1 mm.

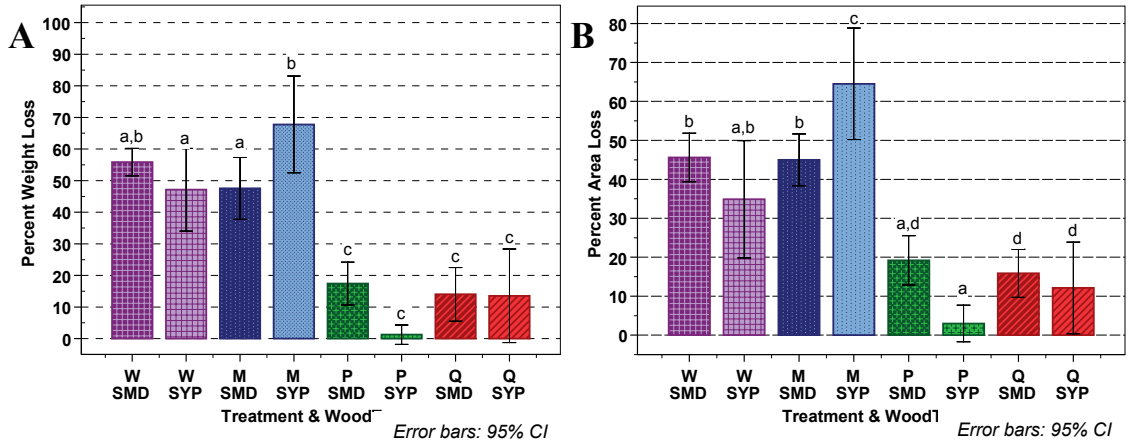

Fig. 12. Comparison of the two wood types across all treatments with regard to (A) mean percent weight loss and (B) mean percent area loss caused by *R. virginicus* feeding during the Live Mycelia Bioassay. Means represented by bars topped with same letters are not significantly different ([A] $F = 36.00$, df = 7, 48, $P < 0.05$; [B] $F = 30.56$, df = 7, 48, $P < 0.05$). M, blank medium control; P, GLT- strain; Q, GLT+ strain; W, distilled water control.

Vacuum Impregnation Bioassay. The weight and area loss measurements taken for the SYP and SMD disks are reported in Figs. 13 and 14, respectively. Regarding SYP, the highest weight loss was seen in disks impregnated with GLT- homogenate (50.87%) and the lowest loss occurred in the distilled water treatment (31.32%). The highest and lowest losses in area also took place in these treatments, respectively. Weight loss was greatest in SMD disks impregnated with blank medium homogenate (48.16%) and least in those treated with the GLT+ strain (40.37%). As with the SYP disks, the area loss in SMD disks closely followed that of weight loss.

The level of correlation between weight and area loss was analyzed. In both wood types, the correlation was significant at $\alpha = 0.01$ (Table 6). The Pearson's correlation coefficient was 0.975 and 0.972 for SYP and SMD disks, respectively. The linear *R* squared value determined for the SYP disks was 0.951 (Fig. 15A). The best fit line on the SMD disks' scatter plot returned a linear *R* squared value equal to 0.946 (Fig. 15B).

Statistically-significant differences ($\alpha = 0.05$) existed among treatments, but only for SYP disks (Table 7); no significant variation was seen in the SMD disks (Table 8). A significant difference occurred between SYP disks impregnated with the GLT- strain and those treated with distilled water (Weight: $F = 3.93$, df = 3, 24, $P < 0.05$; Area: $F = 3.40$; df = 3, 24; $P < 0.05$). SYP disks impregnated with blank medium or GLT+ homogenate were statistically similar in weight and area loss to all treatments tested. SMD disks did not experience any significantly different loss in weight ($F = 0.518$; df = 3, 24; $P = 0.675$) or area ($F = 1.47$; df = 3, 24; $P = 0.252$). Comparisons of the effects experienced by SYP and SMD disks together showed that no statistically-significant differences ($\alpha = 0.05$) existed in either weight loss ($F = 2.03$, df = 7, 48, $P = 0.076$) or area loss ($F = 2.21$, df = 7, 48, $P > 0.053$) (Fig. 16).

Efficacy testing showed that all samples were capable of producing viable *T. virens* cultures on PDA (Fig. 17). Disks subjected to the vacuum impregnation process served as epicenters for *T. virens* growth on PDA (Fig. 17A,B). The liquid samples of GLT+ and GLT- homogenates taken from their respective vacuum chambers following the impregnation process contained viable *T. virens* mycelia and/or spores. PDA inoculated with these samples became completely colonized by *T. virens* within a 5 d period (Fig. 17C,D).

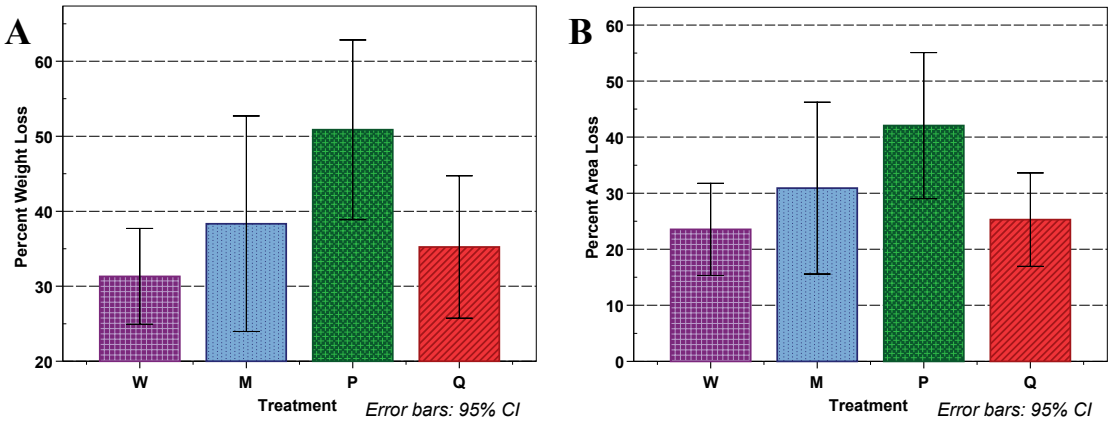

Fig. 13. Consumption of southern yellow pine disks in terms of (A) mean percent weight loss and (B) mean percent area loss by *R. virginicus* during the Vacuum Impregnation Bioassay. M, blank medium control; P, GLT- strain; Q, GLT+ strain; W, distilled water control.

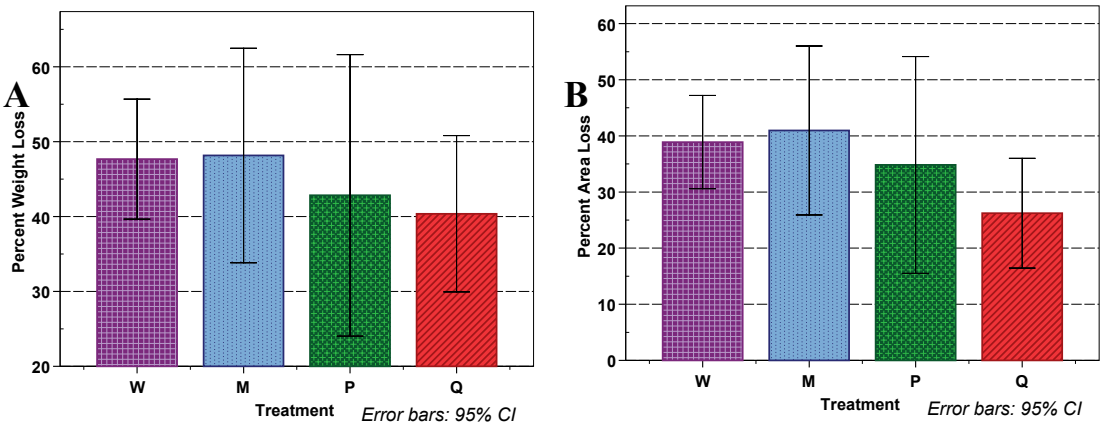

Fig. 14. Consumption of Sentricon® monitoring device disks in terms of (A) mean percent weight loss and (B) mean percent area loss by *R. virginicus* during the Vacuum Impregnation Bioassay. M, blank medium control; P, GLT- strain; Q, GLT+ strain; W, distilled water control.

Table 6. Correlation between weight and area loss in wood disks, regardless of treatment, during the Vacuum Impregnation Bioassay.

		SYP[a]	SMD[b]
Mean ± SD	Weight	38.94 ± 12.28	44.76 ± 12.48
	Area	30.44 ± 12.71	35.22 ± 13.57
Pearson correlation		0.975**	0.972**

** indicates the correlation is significant at the 0.01 level (2-tailed).
[a] SYP, southern yellow pine.
[b] SMD, Sentricon® monitoring device.

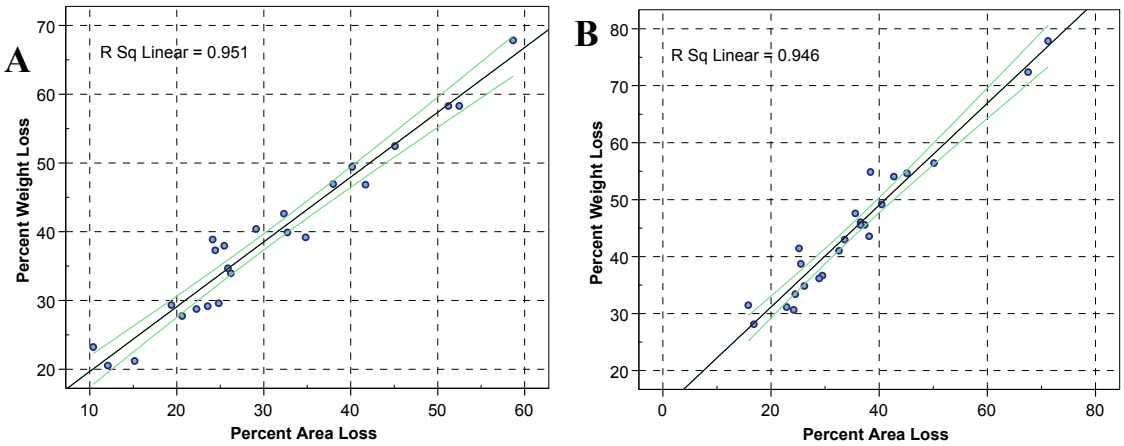

Fig. 15. Scatter plots representing the correlation between weight and area loss of (A) southern yellow pine and (B) Sentricon® monitoring device disks, respectively, used during the Vacuum Impregnation Bioassay.

Table 7. Mean (± SD) percent loss in weight and area of southern yellow pine disks due to *R. virginicus* feeding during the Vacuum Impregnation Bioassay.

Treatment[a]	Percent loss	
	Weight	Area
W	31.32 ± 6.09a	23.55 ± 7.83a
M	38.33 ± 13.69ab	30.90 ± 14.61ab
P	50.87 ± 11.42b	42.05 ± 12.42b
Q	35.24 ± 9.04ab	25.27 ± 7.94ab
F value	3.93	3.40

Means within a column followed by the same letter are not significantly different (α = 0.05; df = 3, 24).
[a] M, blank media control; P, GLT- strain extract; Q, GLT+ strain extract; W, distilled water control.

Table 8. Mean (± SD) percent loss in weight and area of Sentricon® monitoring device disks due to *R. virginicus* feeding during the Vacuum Impregnation Bioassay.

Treatment[a]	Percent loss	
	Weight	Area
W	47.67 ± 7.63a	38.89 ± 7.91a
M	48.16 ± 13.66a	40.96 ± 14.33a
P	42.84 ± 17.91a	34.82 ± 18.40a
Q	40.37 ± 9.94a	26.22 ± 9.32a
F value	0.518	1.47

Means within a column followed by the same letter are not significantly different (α = 0.05; df = 3, 24).
[a] M, blank media control; P, GLT- strain extract; Q, GLT+ strain extract; W, distilled water control.

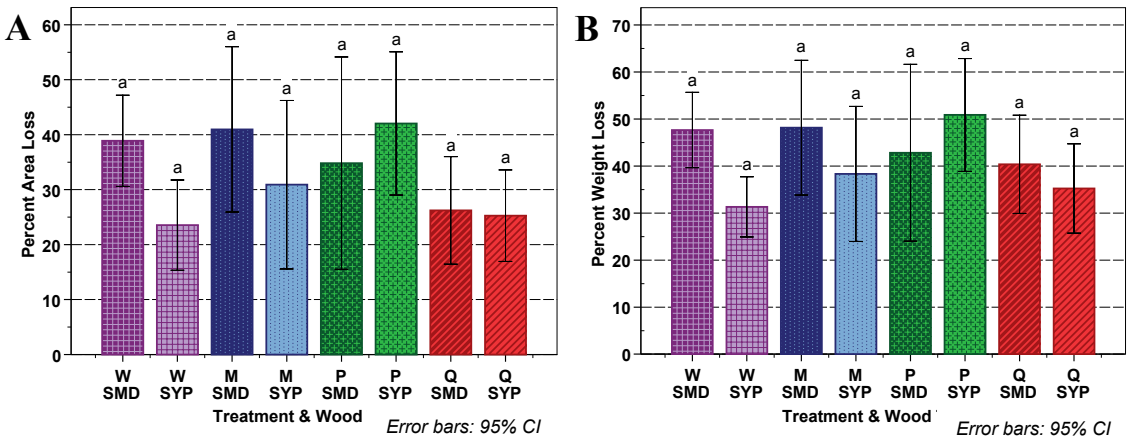

Fig. 16. Comparison of the two wood types across all treatments with regard to (A) mean percent weight loss and (B) mean percent area loss caused by *R. virginicus* feeding during the Vacuum Impregnation Bioassay. Means represented by bars topped with same letters are not significantly different ([A] $F = 2.03$, df = 7, 48, $P > 0.05$; [B] $F = 2.21$, df = 7, 48, $P > 0.05$). M, blank medium control; P, GLT- strain; Q, GLT+ strain; W, distilled water control.

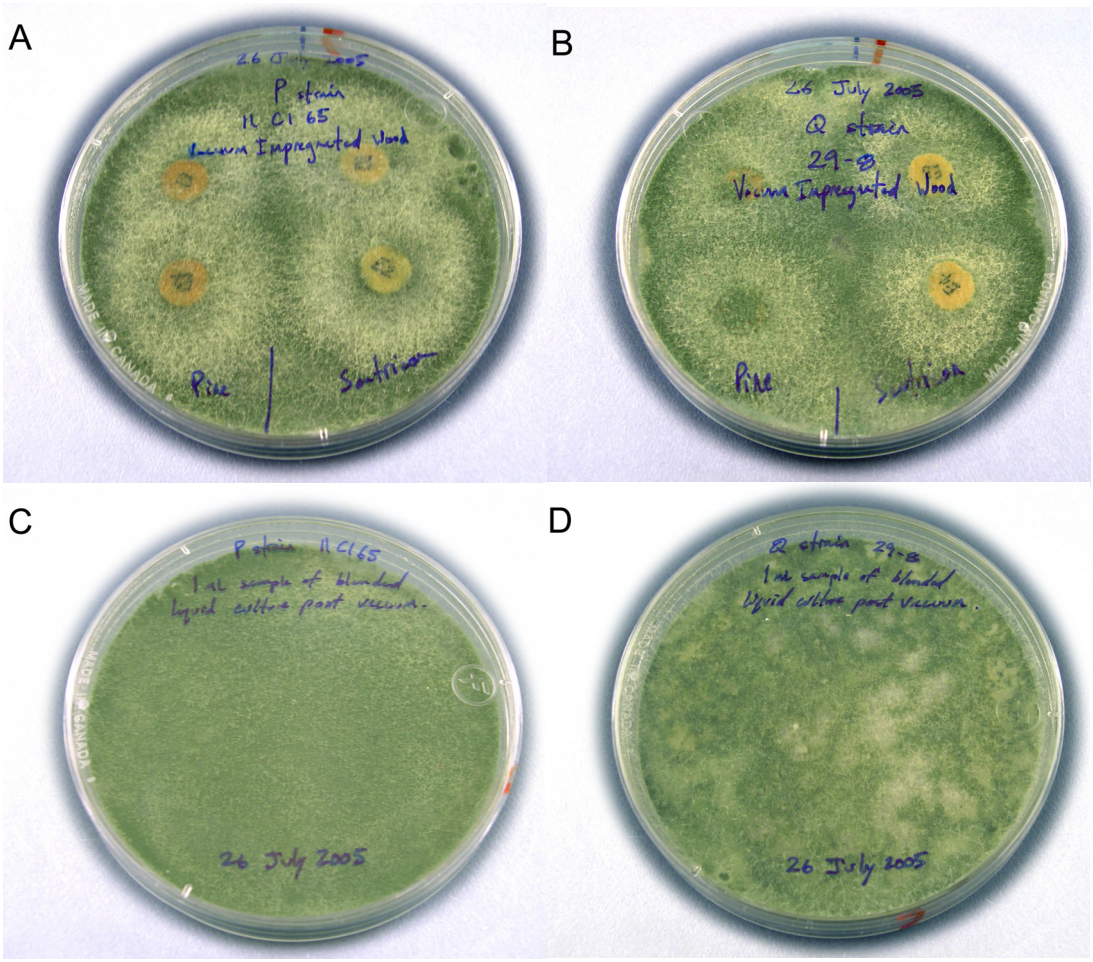

Fig. 17. 5 d old PDA plates inoculated with (A) wood disks impregnated with GLT- strain, (B) wood disks impregnated with GLT+ strain, (C) 1ml sample of homogenated GLT- strain, and (D) 1ml sample of homogenated GLT+ strain all taken from the vacuum chambers after completion of the impregnation process.

Discussion

Fungal Extract Bioassay. The objective of this bioassay was to determine if the extracts taken from the two *T. virens* strains would influence the feeding activity of *R. virginicus* on filter paper disks. In this investigation, *T. virens* extracts regularly had the lowest area loss in filter paper disk consumption by *R. virginicus* (Table 1). The null hypothesis was rejected because significant differences did arise between the fungal extracts and both control treatments

in three separate trials. Results warranted a new hypothesis that certain concentrations of gliotoxin cause decline in *R. virginicus*' cellulose consumption.

Interestingly, in the Day 2 trial the GLT- extract produced significantly more area loss in filter paper disks than both control treatments. Additional experiments would be required to suggest a mechanism for this result. One conclusion that can be drawn is that termites may have fed more in the GLT- treatment due to differences in antibiotics, enzymes, and/or toxins produced by the two fungal strains (Howell et al. 1993). This was the only occasion when a fungal extract treatment caused a significant increase in termite feeding when compared to both controls.

During the Day 6 and Day 7 trials, *R. virginicus* consumed significantly less filter paper in GLT+ extract treatments versus remaining treatments. Filter paper disks treated with GLT+ extracts had 54.78, 53.11, and 41.36% less area loss than disks treated with distilled water, blank medium, and the GLT- strain, respectively. The distinct decline in feeding activity by *R. virginicus* during the two trials, especially Day 7 (Fig. 7), was inversely correlated to the expected rise in gliotoxin levels within the GLT+ extracts. The amount of gliotoxin produced by *T. virens* typically peaks between six and eight days of growth (Park et al. 1991), though the exact time can vary depending on the strain of the fungus and the cultivation substrate (Howell et al. 1993, Anitha and Murugesan 2005).

The medium for this bioassay was similar in composition to that used by Park et al. (1991); therefore, toxin production in the GLT+ strain may have been within its highest range by day seven. Certain fungi are believed to increase their production of antibiotic metabolites during times of nutrient stress, allowing them to suppress nearby competitors (Ghisalberti and Sivasithamparam 1991). In this experiment, the GLT+ strain may have begun exhausting the culture medium of its nutrients within the first 5 d, initiating the production of more toxic

compounds at 6 and 7 d. In theory, when nutrient levels reach critical lows, the fungus would shift its energy allocations from synthesizing toxins to generating chlamydospores for survival. After 15 d in culture, the fungus may have exploited the medium to the point that it began recycling the toxins for additional carbon. This would cause the toxin concentration in the medium to decrease, possibly explaining why area loss in filter paper disks treated with GLT+ extracts in the Day 15 trial returned to levels significantly equal to the controls.

Interactions between termites and fungi are exceedingly unpredictable because of strain variations within a single fungal species. Sibling strains have been reported to induce unique responses in termite behavior (Becker and Kerner-Gang 1964, Lund 1965, Grace et al. 1992, Cornelius et al. 2002), and may be a reason for the contradictory results seen here and in earlier research (Amburgey and Smythe 1977, Grace et al. 1992). In four of the seven trials, a significant difference in area loss was evident when comparing disks treated with the GLT+ strains versus the GLT- strains of *T. virens* (Table 1). In one of those instances, the Day 4 trial, the GLT+ treated filter paper disks endured more area loss (46.60%) than those in the GLT- treatment (21.66%). In the remaining three trials, Day 2, Day 6, and Day 7, *R. virginicus* consumed less of the filter paper disks treated with GLT+ extracts than GLT- extracts. One possible explanation for this is that the strains produced different types and/or concentrations of secondary metabolites, which in turn altered the filter paper's appeal to *R. virginicus*. Again, the metabolite of interest could very well have been gliotoxin because the bioassay tested a gliotoxin-producing GLT+ strain and a non-gliotoxin-producing GLT- strain. These results justify further investigations into gliotoxin's potential to deter termite feeding.

In the future, *T. virens* extracts may be incorporated into a variety of termite management strategies because of their abilities to act as feeding deterrents or attractants. The effects they have on termites depend on the strain and the age of the culture when the extracts are

drawn. This flexibility will allow *T. virens* to be cultured in various manners, so that they can be used either to encourage termite feeding in baiting systems or to repel termites from the wood of structures. Certainly, refinements through comprehensive laboratory research and general fieldwork are needed before *T. virens* extracts can be applied in any termite control system. None-the-less, their potential for use is, at least present, waiting to be developed.

Live Mycelia Bioassay. The experiments done by Highley et al. (1997) showed that *T. virens* metabolites alone were not as effective at preventing wood decay as the living fungus. The objective for the Live Mycelia Bioassay was to determine if the presence of *T. virens* mycelia on wood impacted the feeding activity of *R. virginicus*. Results demonstrated that the presence of mycelia, regardless of *T. virens* strain or the type of wood, significantly decreased the amount of weight and area loss in the wood disks (Fig. 12). Consequently, the null hypothesis for this test was rejected.

After 7 d of growth, mycelia fully covered the top surface and edge of the wood disks. This mycelial mat possibly acted as a temporary physical barrier, preventing the termites from gaining immediate access to the disk. In order to establish contact with the wood, termites were forced to either eat portions of the fungus or lift the disk to get underneath were the mycelia was not as dense. Termites routinely worked their way under the disks in all treatments. Figure 11 represents evidence of feeding from the underside of the disk by showing where the termites consumed the wood, but the mycelia over it remained in place.

Another interesting result involves the consumption of the SYP disks placed on the blank medium. These blank medium control treatments lost significantly more weight and area than the disks in all other treatments, even more than the distilled water controls. This difference may be due to the SYP wood selectively absorbing nutrients and/or liquid from the PDA. These disks did appear to have, and maintain, higher moisture content after being placed on the PDA

for 7 d. Because such differences did not occur with the SMD disks, there may be some physical or chemical aspect of the SYP wood that allowed for these reactions with the PDA.

Applications of *T. virens* in this form certainly would not be the most practical method for preventing the decay of cellulose in termite baiting systems. The obvious reason is that it significantly decreased termite feeding on the wood disks. This effect would certainly change with time, but no extended laboratory or field research has been conducted to assess this situation. In the present bioassay, termites did negotiate through, or around the fungus in order to feed on the wood below. This however, took place under conditions were the termites had no other food available. In nature, termites often forage freely between the multiple sources of food they encounter (Su et al. 1984), and when large sources are found, search tunnel networks decrease (Hedlund and Henderson 1999). During laboratory studies, when wood was removed from feeding arenas, termites resumed their instinctive search for new food sources (Reinhard et al. 1997). Based on this information, if termites did encounter wood covered with *T. virens* mycelia in a bait station, they may leave in search of a more suitable food source.

Vacuum Impregnation Bioassay. The objective of this experiment was to identify whether or not wood vacuum-impregnated with *T. virens* homogenate maintained its palatability to *R. virginicus*. The null hypothesis in this case is rejected for SYP disks (though with some reluctance) and is accepted for SMD disks. The only significant difference in weight and area loss between SYP disks occurred with the GLT- homogenate and the distilled water control. The adjusted *R* squared values for the weight and area loss data sets were both below 0.28. This suggests that the treatment in question may not have been the only cause of the contrast. The lack of any significant differences when all SYP and SMD disks were analyzed together (Fig. 16) is another indication that a strong variation may not exist.

The lack of statistical significance among all treatments may be due more to the experimental design than the treatments themselves. Before blending, distilled water was added to the fungal cultures so that enough liquid would be present to adequately fill the vacuum chambers. This certainly diluted the concentration of secondary metabolites within the mixture. Resulting concentrations may have too low to induce a response from the termites. Though the mycelia remained viable after the vacuum process, the 72 h period of the bioassay may not have allowed sufficient time for metabolite concentrations to rise to effective levels in the wood. This interval was also too brief for the mycelia to completely colonize the disk and provide physical protection from termite feeding.

If this bioassay were to be repeated, at least two major changes could be made to increase its usefulness. First, fungal cultures could be grown in quantities greater than 100 ml. This would eliminate the need to add water before blending, and thus maintain the metabolite concentration of the culture. Second, the duration of the experiment could be extended to a time of 5-7 d. If this were done, either larger pieces of wood should be used or less termites. The combination of these two changes would hopefully create an experimental design that better evaluates the existing differences between treatments.

CHAPTER III

EVALUATION OF SOLDIER LABRAL MORPHOLOGY AS A MEANS OF IDENTIFYING *Reticulitermes* SPECIES (ISOPTERA: RHINOTERMITIDAE) FROM TEXAS

Introduction

Confusion is a feeling many individuals experience when using the subtle differences in soldier morphology to identifying species of *Reticulitermes*. Numerous taxonomic keys have been written covering the *Reticulitermes* spp. of North America (Banks and Snyder 1920, Banks 1946, Weesner 1965, Nutting 1990, Scheffrahn and Su 1994, Hostettler et al. 1995). None of these keys, when describing *Reticulitermes* spp. based on soldier morphology, have been able to avoid the interspecific overlaps in body part shapes and sizes. Subjectivity then becomes a problem, especially when samples are identified without being compared against known specimens. This eventually leads to inaccurate and inconsistent identifications.

Termite identification difficulties have prompted the development of new techniques based on biochemical, chemical, and molecular analyses. These approaches have been run in conjunction with morphological analyses to assess compatibility and consistency (Kaib et al. 1991, Haverty and Nelson 1997, Clément et al. 2001, Foster et al. 2004, Brown et al. 2005). In order to make identifications, alternative techniques must have a baseline or standard by which to evaluate the specimen. Typically, standards are set using characteristics obtained from specimens that fit the original morphological description of the species. Standards based on inadvertent misidentifications can cause significant errors, and may be the reason why results from one technique can contradict those of another.

Perhaps the most reliable and unbiased termite identifications have been made using molecular analyses of mtDNA. Since its incorporation into termite taxonomy by Szalanski et al. (2003), PCR-RFLP has become a valuable means of assessing species synonymy (Austin et al. 2005b, Scheffrahn et al. 2005) and distribution (Austin et al. 2004b, c, d, 2005a). PCR-RFLP and other molecular techniques may benefit termite taxonomy most if they are applied toward re-evaluating the diagnostic morphology of *Reticulitermes*. Molecular assessments have helped clarify the ambiguous morphology within taxa of other organisms, including species of the fungus *Alternaria* (Roberts et al. 2000) and the genus *Prionitis* (Halymeniaceae: Rhodophyta) (Wang et al. 2001).

Three sources of phenotypic variability, the genome, the environment, and mutations, are rooted in the organism's developmental program (Scheiner 1993). A source may act alone, or in combination with another, as the driving force behind variations in species morphology (reviewed by Scheiner 1993, Nijhout 1999, Emlen and Allen 2004). Changing environmental and nutritional conditions in laboratory situations have contributed to morphological and genetic differences among *Drosophila* spp. (Partridge et al. 1994, De Moed et al. 1997, Morin et al. 1997) and *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) (Via and Shaw 1996). Environmental influence on phenotypic expression is also a major aspect in the theory of clinal variations in the morphology of a species. Geographic clines produce differences in physical characteristics of vertebrates and invertebrates (Johnston and Selander 1964, Huey et al. 2000, Chown and Klok 2003). The morphological size difference seen in some *Reticulitermes* spp. from diverse geographic regions (Weesner 1965) is evidence that principles of phenotypic plasticity or clinal variation can be applied to termites.

As phenotypic variations become less distinguishable between termite species, examination of their relation to genetic haplotypes should be required. This would allow for

more accurate species identifications. The objective of this study was to evaluate the feasibility of using soldier labral measurements for the identification of *Reticulitermes* spp. found in Texas. Species identifications using single labrum characteristics (Hostettler et al. 1995) and combinations of multiple characteristics as reported in this thesis were compared against each other, and then with the results of PCR-RFLP analysis. The null hypothesis stating that the identification technique does not reveal significant differences in labral size among *R. flavipes*, *R. hageni*, *R. tibialis*, and *R. virginicus* was tested for the methods described by Hostettler et al. (1995) and additional methods described in this thesis.

Materials and Methods

Termite Collection. Samples of *Reticulitermes* spp. were collected from two Texas cities, College Station and Beeville. *Reticulitermes* samples were acquired in College Station from collection sites at Emerald Forest Park (EFP), Hensel Park (HP), Veterans Park (VMP), and Windwood Park (WWP). These parks were chosen based on the presence of areas conducive to termite activity, as well as their distribution throughout the city. Collection sites within these parks were selected based on discovery of active termite infestations. Multiple collection sites were used at an individual park if suitable conditions existed and large numbers of termites were found in separate areas. At each collection site, in-ground bucket-bait traps were installed and baited as previously described in Chapter II. Termites were collected weekly, as long as activity existed, from each site between June and September 2005. Samples of the soldiers and workers were taken from the traps of active sites and preserved in 95% ethanol. Similar collection methods were employed to obtain *Coptotermes formosanus* Shiraki colonies from Rockport, TX during June 2005. *Coptotermes formosanus* samples were returned to College Station alive before being preserved in 95% ethanol. Additional *Reticulitermes* samples

were gathered on 27 October 2005 from locations within Beeville, TX. These sites included areas of parks and empty lots containing wood debris or dead tree stumps. Collected termites were placed in 95% ethanol for transport back to College Station.

Morphological Assessment. Based on previous identification work by Hostettler et al. (1995), soldier labra were examined for morphometric variations in size and shape. Mature soldiers from each active collection site were selected for inspection. Specimens were removed from ethanol, and allowed to air dry before being decapitated. Under a dissecting microscope (Nikon SMZ-2T, Tokyo, Japan), the labrum of each head capsule was detached from the anteclypeus and mounted on a microscope slide. Polyvinyl alcohol (PVA) mounting medium (#6371, BioQuip Products Inc., Gardena, CA) containing phenol and lactic acid was used to affix the labra to microscope slides. Labra were then photographed at 100X magnification using a digital camera (Olympus DP 11-N C2500L, Tokyo, Japan) and 2.5X lens (Nikon CF PL2.5xA, Tokyo, Japan) mounted atop a compound microscope (Nikon YS2-T, Tokyo, Japan). Photographs were also taken of *R. flavipes* and *R. virginicus* labra voucher specimens that were housed at the Center for Urban and Structural Entomology (Dept. of Entomology, Texas A&M University, College Station, TX).

Images were viewed in SigmaScan® Pro 5 (SPSS Inc. 1999) so that labra lengths and widths could be measured (Fig. 18). Length was measured down the midline from the distal hyaline tip to the point of overlap by the anteclypeus, which is represented by the distinct pigment contrast in the labrum's proximal region. Width measurements were made at the labrum's widest point laterally. A ratio of labral length to width was calculated for each sample using the equation $R_L = L_l / L_w$, where R_L is the labral ratio, L_l is the labral length, and L_w is the labral width. This was repeated with five soldiers from each collection site and both set of *R. flavipes* and *R. virginicus* vouchers.

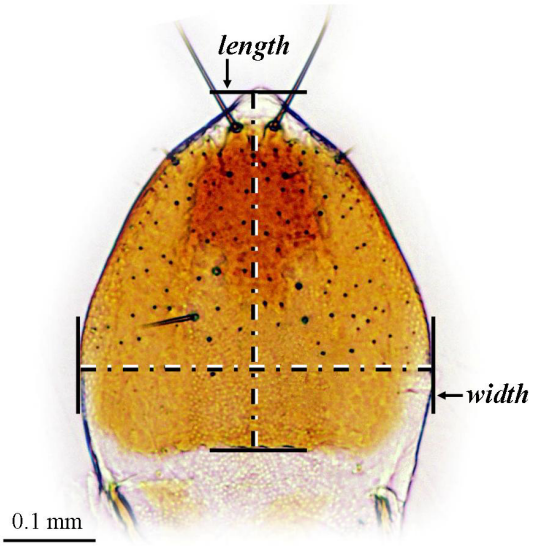

Fig. 18. Labral length when measured from the hyaline tip, down the midline until the sclerotization ended. The width measurement was carried out at the labrum's widest point laterally.

The mean lengths, widths, and length:width ratios for each sample group were statistically analyzed using univariate ANOVA ($\alpha = 0.05$) and Ward's method of hierarchical clustering (JMP IN® 5.1, SAS Institute Inc. 2003). After running ANOVA, means were separated into homogeneous subsets using Tukey's HSD test ($\alpha = 0.05$). ANOVA tests were completed on all measurement data after grouping samples by their respective species. Significant differences that occurred between species were compared using Tukey's HSD test.

Genetic Evaluation. Genetic analysis was conducted to verify the accuracy of the previously described labrum-based identifications. Workers and soldiers from the *Reticulitermes* colonies used in the morphological evaluations were preserved in 95% ethanol and sent to the University of Arkansas (Fayetteville, AR) for DNA extraction. Because termites from Beeville were collected months after those in College Station, the samples were analyzed on separate occasions. Procedures for the molecular analysis were as follows. Alcohol-preserved specimens were air dried on filter paper, and then DNA was extracted according to Liu and Beckenbach

(1992). DNA extractions were made on individual whole worker termites using a Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Tris-EDTA was used to resuspend extracted DNA before storing it at -20ºC. The primers used to amplify an approximately 428-bp region of the mtDNA 16S rRNA gene for the polymerase chain reaction (PCR) were LR-J-13007 (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati and Smith 1995) and LR-N-13398 (5'-CG-CCTGTTTATCAAAAACAT-3') (Simon et al. 1994). PCR reactions were carried out using 1 µl of extracted DNA (Szalanski et al. 2000), having a profile of 35 cycles of 94ºC for 45 s, 46ºC for 45 s, and 72ºC for 60 s. Minicolumns were used to purify and concentrate the amplified DNA from individual termites. Samples of the DNA were then sent to The University of Arkansas Medical Center DNA Sequencing Facility (Little Rock, AR) for direct sequencing in both directions.

Genetic distances, following the Kimura 2-parameter model of sequence evolution (Kimura 1980), were calculated using the distance matrix option of PAUP*4.0b10 (Swofford 2001). Data from the *Reticulitermes* samples collected during this study were compared along side known DNA sequences from *Reticulitermes* and *C. formosanus* previously used by Austin et al. (2004b, c, d). *Coptotermes formosanus* samples from Rockport were not analyzed genetically; instead, the known sequence data of *C. formosanus* from Galveston, TX served as the outgroup taxon. Alignment of the DNA sequences was performed with CLUSTAL W (Thompson et al. 1994). Any gaps in sequence data were treated as missing data when maximum likelihood and unweighted parsimony analysis were run on the alignments. A bootstrap test (Felsenstein 1985), including 1000 resamplings using the Branch and Bound algorithm of PAUP*, was conducted to test the reliability of the parsimony trees.

Results

Morphological Assessment. Length and width of 55 soldier labra were measured and used to calculate length:width ratios (Table 9). Figure 19 shows representative labra from each field-collected colony examined in this study. Statistical differences ($\alpha = 0.05$) were seen among termite samples when labral length, width, or length:width ratio was analyzed. When assessing single characteristics, measurements from the collected sample would frequently overlapped with those from two other sites, and on occasion with three (WWP, length; VMP sites 1 & 2, ratio; *R. flavipes* voucher specimens, ratio) (Fig. 20, Table 9). Measurements of *C. formosanus* soldiers from Rockport were significantly different ($\alpha = 0.05$) from all other samples when comparing length ($F = 32.47$; df = 10, 55; $P < 0.05$), width ($F = 42.13$; df = 10, 55; $P < 0.05$), and ratio ($F = 36.33$; df = 10, 55; $P < 0.05$) (Table 9).

Hierarchical cluster analysis, following Ward's method, was performed on four different combinations of measurement means, including: (1) all three characteristics, length, width, and ratio (Fig. 21A), (2) length and width (Fig. 21B), (3) length and ratio (Fig. 21C), and (4) width and ratio (Fig. 21D). Numbers within the clade indicate the node, which correspond to the distances between clades given in Table 10. Clades generated from the various measurement combinations were constructed differently in each situation; however, certain collection sites were aligned consistently. EFP site 1 samples and *R. virginicus* voucher specimens paired together in all dendograms, as did HP and VMP site 3 termites. Samples from EFP site 7, VMP sites 1 and 2, WWP, and the *R. flavipes* voucher specimens grouped near one another in all four analyses. Only one of the dendograms failed to separate *C. formosanus* from the *Reticulitermes* clades in the initial branch (Fig. 21B).

Table 9. Summary of soldier labral measurements taken from field collected samples and laboratory voucher specimens. Means were determined from the measurements of five slide-mounted labra.

Collection site	City, State	Labral measurement mean ± SD		
		Length[a] (mm)	Width[b] (mm)	Ratio[c]
C. formosanus	Rockport, TX	0.336 ± 0.015a	0.258 ± 0.010a	1.303 ± 0.034a
R. flavipes [d]	CS, TX	0.462 ± 0.027f	0.396 ± 0.030cd	1.167 ± 0.040bcd
R. virginicus [d]	CS, TX	0.425 ± 0.011de	0.420 ± 0.010d	1.011 ± 0.013ef
Emerald Forest Park 1 (EFP1)	CS, TX	0.418 ± 0.007cd	0.399 ± 0.009cd	1.047 ± 0.029f
Emerald Forest Park 7 (EFP7)	CS, TX	0.459 ± 0.011f	0.390 ± 0.007cd	1.177 ± 0.029bc
Hensel Park (HP)	CS, TX	0.390 ± 0.011bc	0.344 ± 0.016b	1.136 ± 0.028cd
Near Tyler & Oak St.	Beeville, TX	0.417 ± 0.016cd	0.347 ± 0.016b	1.203 ± 0.020b
Veterans Park 1 (VMP1)	CS, TX	0.455 ± 0.024ef	0.392 ± 0.023cd	1.161 ± 0.018bcd
Veterans Park 2 (VMP2)	CS, TX	0.459 ± 0.019f	0.395 ± 0.016cd	1.164 ± 0.031bcd
Veterans Park 3 (VMP3)	CS, TX	0.382 ± 0.009b	0.345 ± 0.009b	1.109 ± 0.039e
Windwood Park (WWP)	CS, TX	0.450 ± 0.011def	0.384 ± 0.006c	1.174 ± 0.022bc
F value		32.47	42.13	36.33

Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$; df = 10, 55). CS, College Station; TX, Texas.

[a] Labral length was measured from the hyaline tip, down the midline until the sclerotization ended.

[b] Width measurement was carried out at the labrum's widest point laterally.

[c] Ratio was found by dividing labral length by width.

[d] Voucher specimens were obtained from the Center for Urban and Structural Entomology, College Station, TX.

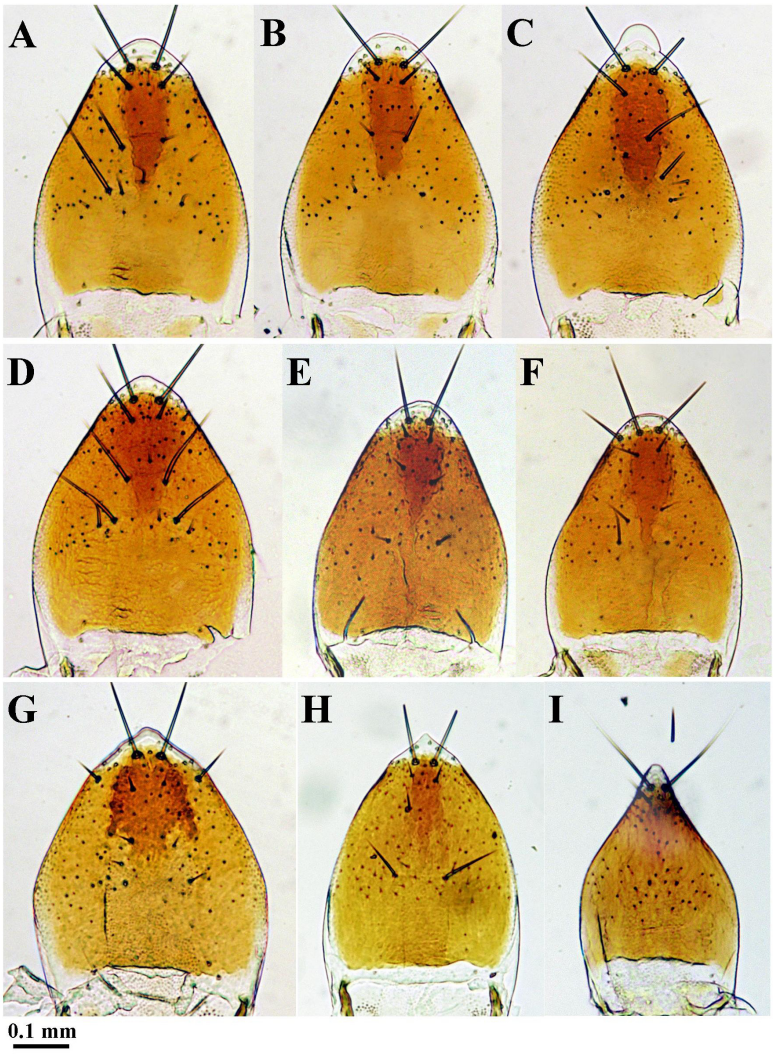

Fig. 19. Slide-mounted labra from the nine field collection sites. Photographs were taken at 100X magnification. Colonies collected in College Station, TX are represented by (A) *R. flavipes* from Veterans Park site 1, (B) *R. flavipes* from Veterans Park site 2, (C) *R. flavipes* from Windwood Park, (D) *R. flavipes* from Emerald Forest Park site 7, (E) *R. hageni* from Hensel Park, (F) *R. hageni* from Veterans Park site 3, and (G) *R. virginicus* from Emerald Forest Park site 1. (H) *R. tibialis* collected in Beeville, TX. (I) *C. formosanus* samples from Rockport, TX served as the outgroup taxon in the morphological portion of this study.

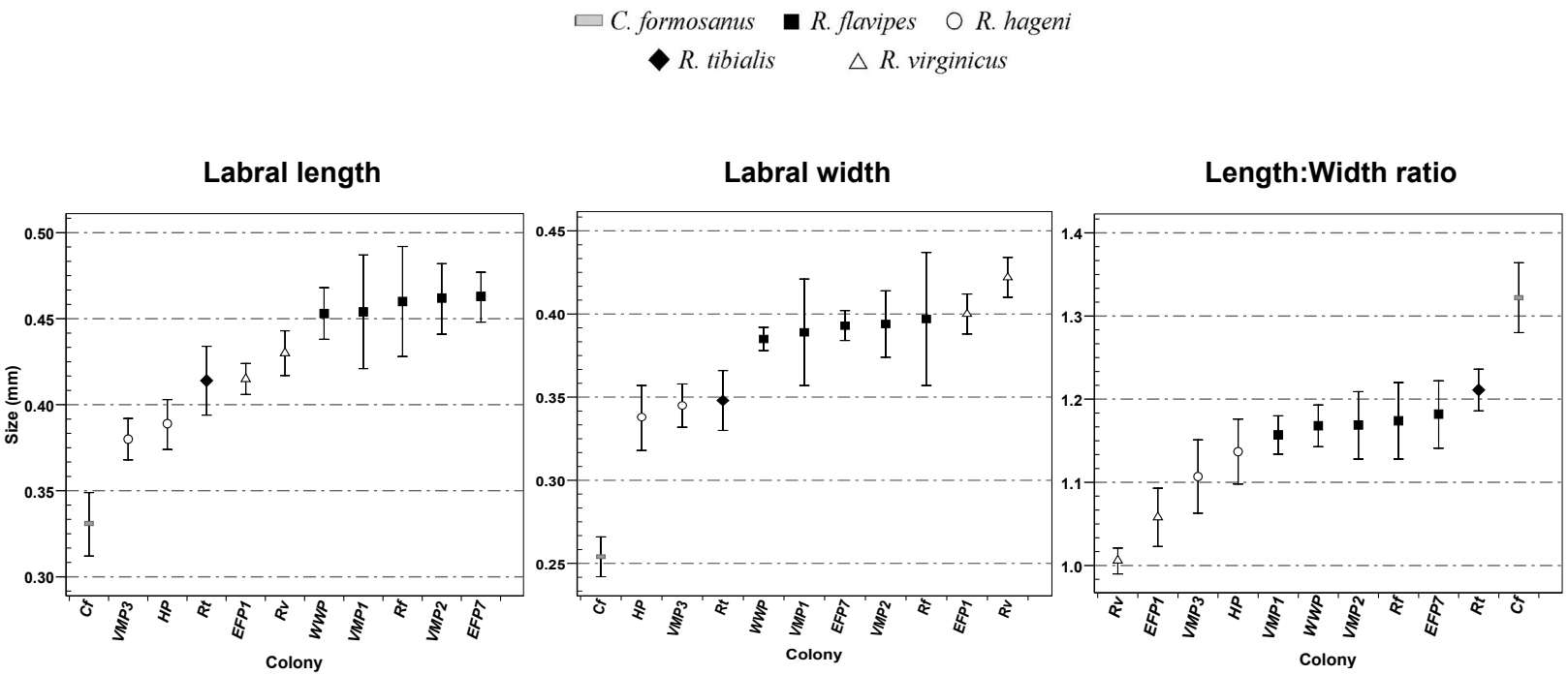

Fig. 20. Mean[a], maximum, and minimum measurements of soldier labra from sampled colonies. Measurements of length and width were performed on five slide-mounted labra per colony. Labral length was divided by width to calculate respective ratios. Cf, *C. formosanus* Rockport, TX; EFP1, Emerald Forest Park site 1; EFP7, Emerald Forest Park site 7; HP, Hensel Park; Rf, *R. flavipes* voucher specimens; Rt, *R. tibialis* Beeville, TX; Rv, *R. virginicus* voucher specimens; VMP1, Veterans Park site 1; VMP2, Veterans Park site 2; VMP3, Veterans Park site 3; WWP, Windwood Park.

[a] Symbols representing species also indicate the respective colony mean on the graphs.

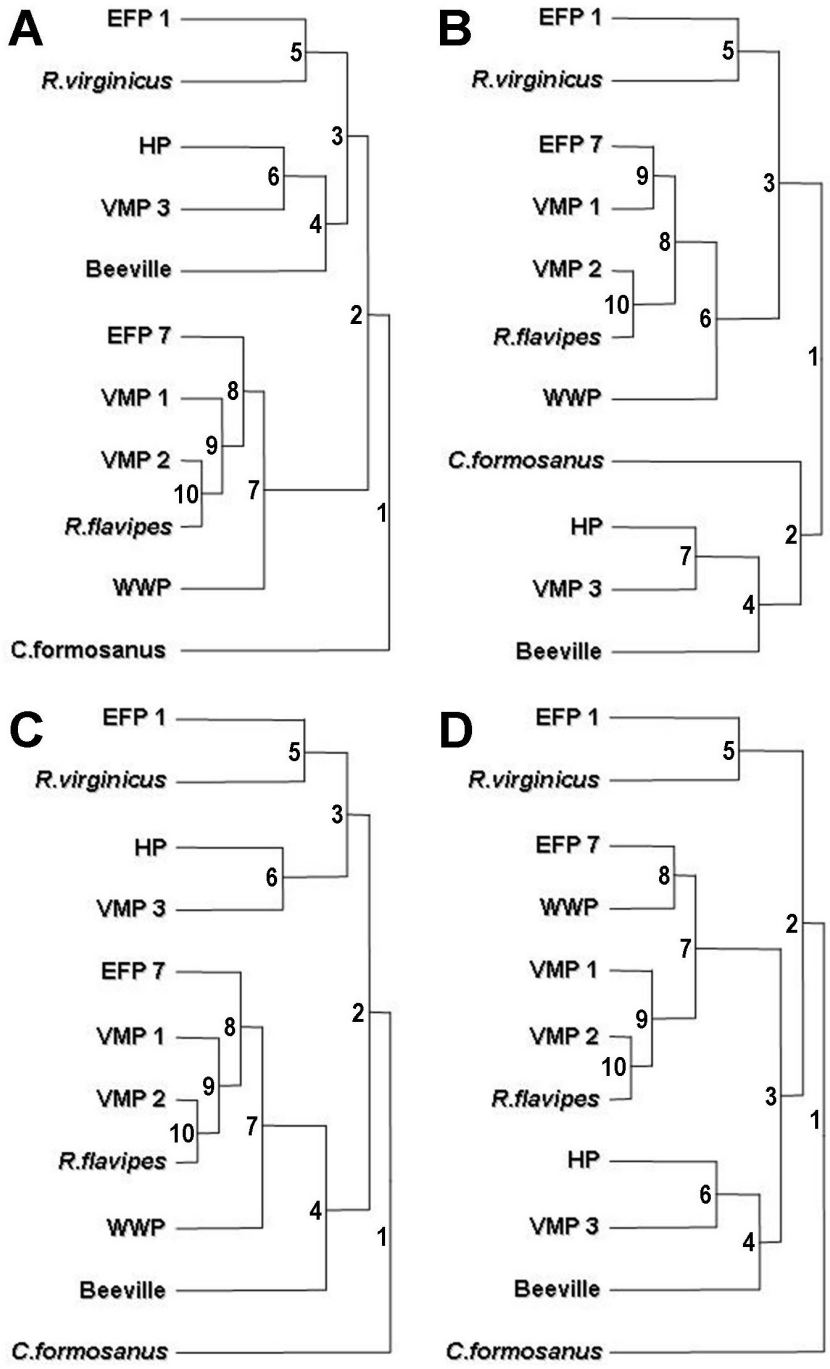

Fig. 21. Dendograms generated from hierarchical cluster analyses (Ward's method) of (A) all three labral measurement data sets (length, width, and ratio), (B) length and width data only, (C) length and ratio data only, and (D) width and ratio data only. The nodes are indicated by the numbers inside the branches. Distances between clusters are represented in Table 10. EFP 1, Emerald Forest Park site 1; EFP 7, Emerald Forest Park site 7; HP, Hensel Park; VMP 1, Veterans Park site 1; VMP 2, Veterans Park site 2; VMP 3, Veterans Park site 3; WWP, Windwood Park.

Table 10. Distances between clusters in dendograms of Fig. 21A-D. Values were calculated using Ward's linkage function.

Length, width, and ratio[a] corresponds to Fig. 21A				Length and width[a] corresponds to Fig. 21B			
Node[b]	Leader	Joiner	Distance	Node[b]	Leader	Joiner	Distance
10	VMP 2	*R.flavipes*	0.059	10	VMP 2	*R.flavipes*	0.050
9	VMP 1	VMP 2	0.138	9	EFP 7	VMP 1	0.079
8	EFP 7	VMP 1	0.163	8	EFP 7	VMP 2	0.130
7	EFP 7	WWP	0.276	7	HP	VMP 3	0.144
6	HP	VMP 3	0.286	6	EFP 7	WWP	0.265
5	EFP 1	*R.virginicus*	0.484	5	EFP 1	*R.virginicus*	0.350
4	HP	Beeville	1.063	4	HP	Beeville	0.638
3	EFP 1	HP	2.414	3	EFP 1	EFP 7	1.164
2	EFP 1	EFP 7	2.516	2	*C.formosanus*	HP	2.124
1	EFP 1	*C.formosanus*	4.034	1	EFP 1	*C.formosanus*	3.672

Length and ratio[a] corresponds to Fig. 21C				Width and ratio[a] corresponds to Fig. 21D			
Node[b]	Leader	Joiner	Distance	Node[b]	Leader	Joiner	Distance
10	VMP 2	*R.flavipes*	0.055	10	VMP 2	*R.flavipes*	0.040
9	VMP 1	VMP 2	0.122	9	VMP 1	VMP 2	0.078
8	EFP 7	VMP 1	0.142	8	EFP 7	WWP	0.106
7	EFP 7	WWP	0.200	7	EFP 7	VMP 1	0.239
6	HP	VMP 3	0.286	6	HP	VMP 3	0.248
5	EFP 1	*R.virginicus*	0.354	5	EFP 1	*R.virginicus*	0.469
4	EFP 7	Beeville	0.984	4	HP	Beeville	0.851
3	EFP 1	HP	1.489	3	EFP 7	HP	1.446
2	EFP 1	EFP 7	2.665	2	EFP 1	EFP 7	2.382
1	EFP 1	*C.formosanus*	3.070	1	EFP 1	*C.formosanus*	3.340

EFP 1, Emerald Forest Park site 1; EFP 7, Emerald Forest Park site 7; HP, Hensel Park; VMP 1, Veterans Park site 1; VMP 2, Veterans Park site 2; VMP 3, Veterans Park site 3; WWP, Windwood Park.

[a] Combination of labral measurements analyzed by hierarchical cluster analysis.

[b] Numbering coincides with the nodes indicated in the dendograms of Fig. 21.

Genetic Evaluation. The molecular procedures outlined by Szalanski et al. (2003) were used as the basis for sample identifications (Table 11). PCR-RFLP analysis of the mtDNA 16S rRNA amplicon revealed average base frequencies of A = 0.41, C = 0.23, G = 0.13, and T = 0.23. Phylogenetic relationships between all eight collection site samples and the outgroup taxon, *C. formosanus*, were ascertained through bootstrap analysis of the aligned DNA sequences. This process used 449 characters, of which, 30 (7%) were variable and 54 (12%) were constant, to create a single consensus tree with distinct branches separating species (Fig. 22). Clade divergence occurred between *R. flavipes*, *R. hageni*, *R. tibialis*, and *R. virginicus* samples. No genetic structuring was observed between the haplotypes of individual *Reticulitermes* spp.

With identities known, labra measurements from collection sites were compiled by their respective species. Statistical evaluations of independent measurement means indicated significant overlap ($\alpha = 0.05$) among species (Table 12). The only species having significant differences from all others, regardless of the measurement, was *C. formosanus*. At least two *Reticulitermes* spp. were statistically similar in any one measurement category. When analyzing labral length, *R. tibialis* and *R. virginicus* showed no significant differences ($F = 86.17$; df = 4, 55; $P = 0.987$) in their mean value. Analysis of labral width showed no significant difference between *R. hageni* and *R. tibialis* ($F = 102.54$; df = 4, 55; $P = 0.997$). No significant difference occurred between *R. flavipes* and *R. tibialis* ($F = 86.73$; df = 4, 55; $P = 0.131$) when analyzing labral length:width ratios. Comparisons of maximum and minimum labral measurement values also manifested overlaps among the *Reticulitermes* spp. (Table 12). Again, *C. formosanus* had the only labral measurements that did not coincide with another species.

After the termite samples were identified genetically, the maximum parsimony tree was compared to the hierarchical cluster analysis dendograms generated using combinations of labral

measurements. The arrangement of samples into species clades in the parsimony tree coincided most with the dendogram based on labral length and length:width ratio (Fig. 23). Collection sites where samples of the same species were found also separated from other species in similar patterns in the parsimony tree and dendogram. The remaining dendograms based on other combinations of labral measurements divided species clades in ways that did not concur with the parsimony tree. Cluster analysis of all three labral measurements (Fig. 21A) the Beeville sample representing *R. tibialis* grouped nearest the *R. hageni* and *R. virginicus* samples. The dendogram for labral length and width (Fig. 21B) did not show *C. formosanus* as an outgroup taxon. When labral width and length:width ratio were analyzed (Fig. 21D), *R. hageni* samples were more closely correlated with *R. flavipes* samples.

Table 11. Identification of the species collected at each field site. Identifications were made using 16S rRNA gene analysis. Locality information for field sites is also provided.

Species	Collection Site	City	County	State
R. flavipes	Emerald Forest Park site 7 (EFP7)	College Station	Brazos	TX
	Veterans Park site 1 (VMP1)	College Station	Brazos	TX
	Veterans Park site 2 (VMP2)	College Station	Brazos	TX
	Windwood Park (WWP)	College Station	Brazos	TX
R. hageni	Hensel Park (HP)	College Station	Brazos	TX
	Veterans Park site 3 (VMP3)	College Station	Brazos	TX
R. tibialis	Near Tyler and Oak Streets	Beeville[a]	Bee	TX
R. virginicus	Emerald Forest Park site 1 (EFP1)	College Station	Brazos	TX

[a] Beeville, TX is the location from which *R. tibialis* was first collected and described by Banks (Banks and Snyder 1920).

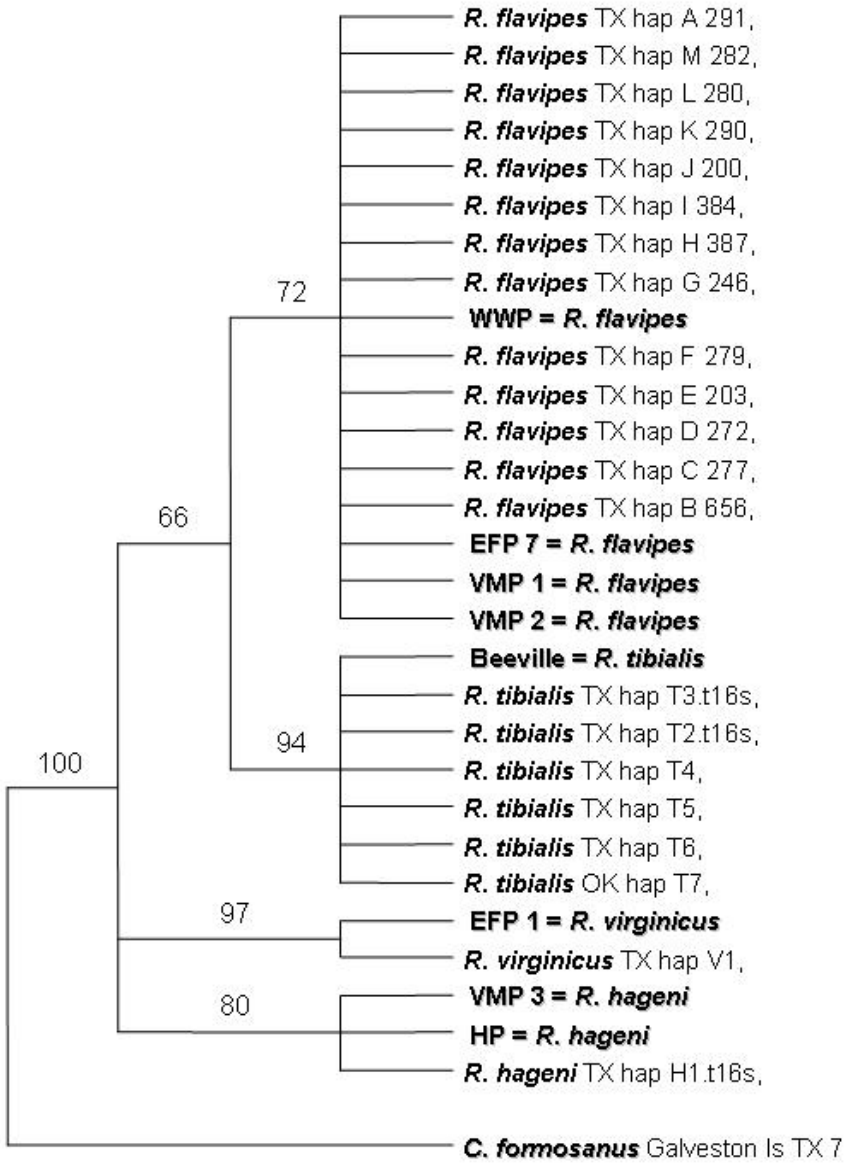

Fig. 22. Single most parsimonious tree based on mtDNA 16S rRNA gene sequences during a branch and bound search with PAUP*. Bootstrap percentages for 1000 iterations are listed above the branches supported at ≥ 50%.

Table 12. Labra measurements assembled based on their respective species, as identified using PCR-RFLP analysis of the mtDNA 16S rRNA gene.

Species	n	Length[a] (mm)			Width[b] (mm)			Ratio[c]		
		Max	Min.	Mean ± SD	Max	Min.	Mean ± SD	Max	Min.	Mean ± SD
C. formosanus	5	0.349	0.312	0.336 ± 0.015a	0.266	0.242	0.258 ± 0.010a	1.364	1.280	1.303 ± 0.034a
R. flavipes	25	0.492	0.421	0.457 ± 0.018b	0.437	0.357	0.391 ± 0.018b	1.222	1.128	1.168 ± 0.027b
R. hageni	10	0.403	0.368	0.386 ± 0.010c	0.358	0.318	0.344 ± 0.012c	1.176	1.063	1.122 ± 0.035c
R. tibialis	5	0.434	0.394	0.417 ± 0.016d	0.366	0.330	0.347 ± 0.016c	1.236	1.186	1.203 ± 0.020b
R. virginicus	10	0.443	0.406	0.422 ± 0.010d	0.434	0.388	0.410 ± 0.014d	1.093	0.990	1.029 ± 0.029d
F value				86.16			102.54			86.73

Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$; df = 4, 55).
[a] Labral length was measured from the hyaline tip, down the midline until the sclerotization ended.
[b] The width measurement was carried out at the labrum's widest point laterally.
[c] Ratio was found by dividing the labrum's length by its width.

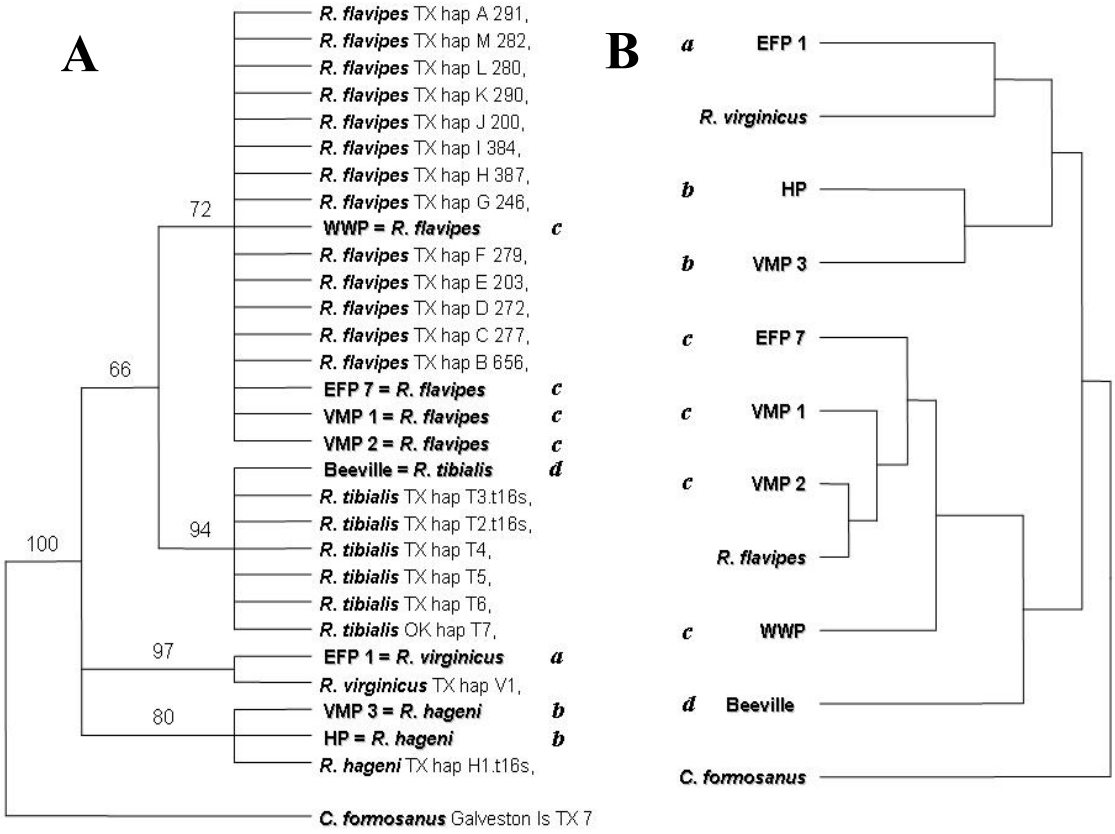

Fig. 23. Comparison of (A) the single most parsimonious tree based on mtDNA 16S rRNA gene sequences during a branch and bound search with PAUP* against (B) the hierarchical dendogram for labral length and length:width ratio measurements generated through Ward's method of cluster analysis. Bootstrap percentages listed above the branches in the parsimony tree are supported at ≥ 50% for 1000 iterations. Field collection sites with the same lowercase letters indicate the same species. EFP 1, Emerald Forest Park site 1; EFP 7, Emerald Forest Park site 7; HP, Hensel Park; VMP 1, Veterans Park site 1; VMP 2, Veterans Park site 2; VMP 3, Veterans Park site 3; WWP, Windwood Park.

Discussion

This study evaluated the effectiveness of using soldier labral characteristics for identification of the four *Reticulitermes* species found in Texas. In order to draw accurate conclusions about the techniques, samples were also identified through molecular analysis of the mtDNA 16S rRNA gene. During this investigation, the use of single labral characteristics failed to show significant differences among each species of *Reticulitermes*. Alternatively, the

combination of length and length:width labral measurements produced significant differences among the four species of *Reticulitermes*. This technique also arranged the sampled species into clades that correlated to those generated during molecular analysis. These results also provided evidence that phenotypic variations seen in termites may parallel the haplotype diversity.

Shape was ruled out as a definitive means for species recognition in the sampled termites. Shape differences similar to those mentioned by Hostettler et al. (1995) did exist in the labra samples from *Reticulitermes* spp. collected during this study; however, they could not be used as an independent method of species identification. The slight differences in labral shapes were subjective, especially when attempting to identify specimens individually. Using labral shapes to distinguish among species was only practical when specimens were compared side-by-side. The problems of using these shape differences for species identifications were compounded by intraspecific variations (Fig. 19A-F). Genetic shifts, environmental influences, or mutations can be responsible for causing the divergence in labral phenotypes seen in these samples (Scheiner 1993).

After identifying the termite samples genetically, labral measurements were reviewed to determine if individual labral characteristics (length, width, and length:width ratio) were significantly different among all the sampled species. No characteristics were significantly different among all species; therefore, the null hypothesis was accepted. Colony maximum and minimum measurements for all three labral characteristics overlapped considerably in the *Reticulitermes* spp. (Fig. 20, Table 12). An underlying reason for this may be that haplotype changes are causing the expression of phenotypic variance. Size deviations may also result from random changes in the internal developmental pathways that workers and/or larva experience while molting into soldiers. This would explain why termites, despite their genetic similarity within colonies, are still able to show such phenotypic variation.

A combined assessment of labral length, and length:width ratio proved to be the most reliable method for separating the species of *Reticulitermes* used in this study. When these data were compiled in a cluster analysis, resulting clade arrangements mirrored those generated through bootstrap analysis of mtDNA 16s rRNA gene sequences (Fig. 23). All *Reticulitermes* spp. separated into unique clades at the third level nodes (Fig. 23B). Clades of *R. virginicus* and *R. hageni* grouped closest to each other and were separated from *R. flavipes* and *R. tibialis* by supported nodes early in both analyses. This is expected, based on morphological size similarities, and molecular studies that consistently separate these termite species (Jenkins et al. 2000, Austin et al. 2002, 2004a, b, c, d; Foster et al. 2004). The internal separation of WWP *R. flavipes* from those of EFP site 7, VMP site 1, and VMP site 2 in the morphology-based dendogram was maintained in the parsimony tree by the extended distances between these groups (Fig. 23A). This suggests that, within *Reticulitermes* spp., labrum phenotypes follow similar variation patterns as genetic haplotypes.

Significant differences did not exist among all specimens of this investigation when labral characteristics currently used to make species identifications were used individually. This is not an attempt to discredit the previous findings of Hostettler et al. (1995), it is instead meant to call attention to the phenotypic diversity within termite species. Geographic restrictions accompany any morphology-based taxonomic key, and identifications using them should be done so with prudence. Perhaps the combination of molecular and morphometric analysis used in this study can be applied to additional termite characteristics (pronotal dimensions, head capsule size, mandible curvature, etc.) in order to increase identification accuracy. Expansion of these techniques could aid in assessing phenotypic plasticity and clinal variation within *Reticulitermes*, allowing for a more accurate revision of the genus.

CHAPTER IV

CONCLUSIONS

Trichoderma virens and Southeastern Subterranean Termites, *Reticulitermes virginicus*

The effects fungi have on termites are dependent on numerous variables. Fungi, termites, and the environment all possess influential characteristics that alter their association with one another. Subterranean termites routinely encounter numerous species of fungi while foraging for food (Hendee 1933, Waller et al. 1987, Zoberi and Grace 1990), and undoubtedly, compete with several of these species for available cellulose resources. Research has proven that termites react differently to a fungus based on the fungal age, strain, and even the manner by which they encounter the fungus (Becker and Kerner-Gang 1964, Lund 1965, Grace et al. 1992, Cornelius et al. 2002). The three bioassays completed for this study, when assessed as a whole, support the broad nature of termite-fungal relationships.

The first bioassay evaluated the feeding of *R. virginicus* on filter paper inoculated with *T. virens* extracts. The focus was to determine how secondary metabolites produced in *T. virens* affected termite feeding. Outcomes varied, depending on the age of the fungus at the time an extract was taken. From a control standpoint, the most interesting results took place in the Day 2, Day 6, and Day 7 trials. Extracts harvested from the GLT- strain on the second day of culture growth actually increased termite feeding by as much as 11% over the control. An opposite effect was observed in the Day 6 and 7 trials, where extracts from the GLT+ strain of *T. virens* significantly decreased termite consumption (54% in Day 7). In order to state definitively that gliotoxin was the true factor in causing the termites to consume less filter paper, pure samples of

the toxin would need to be tested. A hypothesis can be made that filter paper inoculated with gliotoxin is less palatable to *R. virginicus* than blank filter paper. Future research to examine the ability of *T. virens* to influence behaviors in *Reticulitermes* spp. is warranted. The results of this bioassay justify a more in-depth investigation into the use of gliotoxin to curb cellulose ingestion by termites.

The second bioassay tested how *R. virginicus* reacted to wood covered in *T. virens* mycelia. Presence of mycelia, regardless of the *T. virens* strain, significantly decreased wood consumption by termites. Weight loss experienced by treated wood was at least 33% less in SMD disks and 30% less in SYP disks. The wood was exposed to the fungus for a seven day period before being offered to termites. Based on the results of the first bioassay, only the GLT+ strain was expected to decrease feeding. However, both fungal strains had a similar effect, indicating the mycelia may have acted more as a physical barrier than a source of unpalatable chemicals. This was substantiated when close examination of the wood disks revealed termite feeding underneath fungal mycelia. Termites were observed ingesting the fungus, but only in an effort to access the wood. These results indicate that mycelia of *T. virens* had an ability to control termite feeding, but the practicality surrounding an application method is a major hurdle. The mycelia's physical principles may serve as a model for developing new, synthetic barriers that work against termite activity.

The third bioassay investigated the feeding response which *R. virginicus* had to wood vacuum-impregnated with *T. virens* homogenate. The vacuum impregnation process was designed to yield a wood disk treated externally and internally with the fungus. No clear control advantage resulted from the vacuum impregnation of fungal homogenate into the wood. Consumption levels were statistically similar among treatments and controls for both wood types. Whether this means that *T. virens* homogenate can be used effectively in termite bait

stations to control the growth of wood decay fungi while maintaining the wood's attractancy to termites, cannot be entirely assessed by this test. One adjustment that could be made is to incubate inoculated disks for a shorter period before feeding them to termites. This would allow *T. virens* to begin colonizing the wood. Increasing the size of the wood is a simple alternative that would extend the length of the experiment, thus allowing the fungus more time to grow. These changes in experimental design may be necessary to better evaluate this inoculant form.

Two methods used in this research could be assimilated into a variety of feeding studies; therefore, special attention should be directed to them. The vacuum impregnation technique from the third bioassay proved to be a sufficient method of inoculating wood with liquids. Submitting wood disks to vacuum treatment for as little as 4 h caused more thorough penetration of liquids than a soaking lasting twice as long. Future feeding and toxicity studies should be able to adapt the described vacuum system as a means of thoroughly treating wood with various soluble solutions. The second method was the substitution of area loss quantifications via digital images for weight loss calculations. This technique, in slightly altered systems, has been proven effective in the past (Su and Messenger 2000, Vahabzadeh 2002). The combination of camera equipment, analysis software, and protocol permitted calculations of area losses that correlated with their respective weight losses by an average linear *R* squared value of 0.951. Benefits of the digital analysis surface when data need to be collected from small objects of very little weight (such as the filter paper and wood disks evaluated here). Weighing objects is not always feasible during bioassays; in these situations, digital analysis of area loss would be an alternative method for recording data.

A goal of this project was to uncover a biocontrol agent that could be incorporated into termite baiting systems to reduce the presence of wood-rotting fungi. Maintaining the bait systems efficacy is critical, and any biocontrol agent utilized would have to be impartial in the

least toward termite activity. In an ideal situation, the biocontrol agent would act synergistically with the bait's appeal and palatability. The *T. virens* strains tested here showed the capacity to induce neutral, positive, and even negative feeding responses in at least one *Reticulitermes* species. In terms of production and application, the extracts taken from *T. virens* cultures were easier to work with and seem the more practical candidate out of the three forms tested to be integrated in to termite baiting systems.

One major issue not addressed by this research was the application of these *T. virens* forms in field trials. Understandably, field experiments produce results that are more applicable to natural situations than laboratory tests, but a baseline of knowledge must be set first. In the laboratory, the ability of *T. virens* extracts to inhibit termite feeding is a promising step toward biocontrol. However, the methods described here may be just as promising for later research on termite-fungal interactions, because, as Esenther and Beal (1979) stated, "the best fungus … will differ depending on the termite species and perhaps also geographical location." To date, no reported evaluations exist regarding the influence multiple forms of *T. virens* have on the feeding behaviors of *R. virginicus*. These findings are meant to serve as a foundation from which further studies, including field applications, can advance.

Classification of *Reticulitermes* Species by Labral Morphology

The need to describe, identify, and arrange organisms into a taxonomic structure is a trait exemplified by the human species. The methods used to determine classification schemes have become more advanced in the recent years, making once difficult identifications possible. Some of the stressful situations caused by the vague morphology of certain species have been alleviated by the progression in areas of biochemistry and molecular science. Applications of these techniques in entomology have helped refine the taxonomy of termites. The work

described in this thesis demonstrates how molecular analyses can be used to validate the diagnostic morphology of termite soldiers.

An evaluation of the labral characteristics (length, width, and length:width ratio) currently relied upon for definitive identification of termite species (Hostettler et al. 1995) was made using *C. formosanus* and the four *Reticulitermes* spp. found in Texas at present. When assessed individually, these traits consistently isolated *C. formosanus*, but failed to separate the four *Reticulitermes* spp. into significantly different groups. The species paired together were not uniform across all three traits. These results may appear to contradict the findings of Hostettler et al. (1995); but, when *R. tibialis* (which was excluded by Hostettler et al. [1995] because the species does not occur in Florida) is eliminated from the group, the length:width ratio effectively separated the remaining three *Reticulitermes* species.

Since the overlap in distribution of these four *Reticulitermes* spp. is unique to the south central United States (Austin 2005), there is an important need to create diagnostic descriptions of these species. The evaluation of labral morphology presented in this thesis found that combinations of characteristics must be examined for an accurate separation of *R. flavipes*, *R. hageni*, *R. tibialis*, and *R. virginicus*. By analyzing labral length, along with the length:width ratio, all subterranean termite species found in Texas can be positively identified. Different combinations of characteristics did result in species segregations, but only the length and length:width ratio combination correlated well with the molecular diagnostics.

The confidence on which this research is based would not have been as strong without the aid of molecular diagnostics. Application of the PCR-RFLP methods developed by Szalanski et al. (2003) furnished dependable species identifications from which morphological results could be graded. The precision of basing termite identifications on mtDNA 16S rRNA gene sequences is extensively covered by Austin (2005). If the accuracy of this technique is

used to support future evaluations of termite morphology, even the most diagnostically problematic genera could be reliably revised. One caveat to such a revision is the need for wide-range sampling across each species' known distributions. Work of this nature is necessary because of the genotypic and phenotypic variability seen throughout termite species.

Shifts in size among animals are routinely a result of environmental stresses (Partridge et al. 1994, Via and Shaw 1996, De Moed et al. 1997, Morin et al. 1997) and overall clinal variation (Johnston and Selander 1964, Huey et al. 2000, Chown and Klok 2003). *Reticulitermes flavipes* is able to prosper in an extensive habitat range covering North America, South America, and Europe (Austin et al. 2005b). Because distinct climatic diversity exists within this geographic range, variations in morphological characteristics are expected. Inside the limited spatial area sampled for this study, discrepancies between *R. flavipes* colonies were noticed. These phenotypic variances interestingly mirrored the haplotype differences within *R. flavipes*, which further supports corroborating morphological analyses with molecular diagnostics.

As alluded to earlier, the taxonomy of the *Reticulitermes* genus, and possibly many other termite genera, could be revised more effectively if a coordinated analysis of morphology and genetics is conducted. This would certainly be a long and arduous task for anyone to surmount, but the convoluted morphology alone makes this job inevitable. The primary purpose of this research was to investigate the reliability of using morphological characteristics of soldier labra to separate the four *Reticulitermes* spp. found in Texas and to validate the identifications through molecular analyses of the mtDNA 16S rRNA gene. In doing so, these efforts illustrated how worthwhile a multi-angled approach to analysis is for correcting termite identification methods.

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