Comparison of Subterranean Termite (Rhinotermitidae: *Reticulitermes*) Gut Bacterial Diversity Within and Between Colonies and to Other Termite Species Using Molecular Techniques (ARDRA and 16S rRNA Gene Sequencing)

Marc Lewis Fisher

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Entomology

APPROVED:

Dini M. Miller
(Co-Chair)

Carlyle C. Brewster
(Co-Chair)

Donald E. Mullins
Allan W. Dickerman
Claudia Husseneder

April 25, 2006
Blacksburg, VA

Keywords: Bacteria, Diversity, Symbiosis, Termites, ARDRA, 16S rRNA

Copyright 2006, Marc L. Fisher

Comparison of Subterranean Termite (Rhinotermitidae: *Reticulitermes*) Gut Bacterial Diversity Within and Between Colonies and to Other Termite Species Using Molecular Techniques (ARDRA and 16S rRNA Gene Sequencing)

Marc Lewis Fisher

Abstract

Termites are known to harbor within their gut a diverse assemblage of symbiotic microorganisms. Little work has been done, however, to describe the diversity and function of the bacteria in the economically important eastern subterranean termite, *Reticulitermes flavipes*.

The first object of this study was to characterize the bacterial diversity in the gut of *R. flavipes* using amplified rDNA restriction analysis (ARDRA) and 16S rRNA gene sequencing. It was determined that ARDRA was an effective technique for characterizing the diversity of the termite gut microbiota. Of the 512 clones analyzed in the ARDRA study, 261 different ARDRA profiles were found. Forty-two 16S rRNA gene sequences were also analyzed, resulting in 33 different ribotypes. Representatives from six major bacterial phyla, *Proteobacteria*, *Spirochaetes*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and the newly proposed "*Endomicrobia*," were discovered. Further analysis indicated that the gut of *R. flavipes* may harbor as many as 1,318 ribotypes per termite.

The second objective was to determine if the gut bacterial diversity could be manipulated by changing the termite's food source. Using ARDRA analysis, I found no evidence that changing the food source affected the termite gut bacterial diversity. In

addition, changing the food source did not induce aggression in nestmates fed on different food sources.

The third objective was to search for patterns of coevolution between termites and their gut symbiotic bacteria. Using rRNA gene sequences from this study and sequences from public databases (1,450 sequences total), a neighbor-joining tree demonstrated strong evidence for coevolution of termites and their symbiotic bacteria, especially in the phyla *Bacteroidetes*, *Actinobacteria*, *Spirochaetes*, and "*Endomicrobia.*" Many monophyletic clusters were entirely composed of phylotypes specific to Isoptera.

Dedication

I dedicate this work to my wife, Sarah I. Fisher, and my children, Avanlee K. Fisher, Dallin J. Fisher, Savannah J. Fisher, Christian J. Fisher, and Liriel M. Fisher. They have sacrificed a great deal of time, money, and energy supporting their father through his graduate education.

Acknowledgements

With great appreciation I thank my co-advisors, Drs. Dini Miller and Carlyle Brewster. They let me try new things, allowed me to make mistakes and learn from them, and never doubted my ability to succeed. They have always been very supportive financially, emotionally, and intellectually. I am also grateful for my committee members, Drs. Don Mullins, Allan Dickerman, and Claudia Husseneder. Drs. Dickerman and Husseneder provided precious laboratory space and financial assistance that made this study possible. Dr. Mullins was always willing to sit down and give sage advice. I also want to thank the dean of the Virginia Tech Graduate School, Dr. Karen DePauw. She provided timely assistantship support throughout my final two years of graduate school.

I thank Elena Shulaeva for her assistance in the VBI laboratory and Billy Wise, from the Louisiana State University Agricultural Center, for his perseverance in helping me complete the sequencing.

This work was partially funded by grants and scholarships from the Virginia Tech Graduate Student Assembly, the Entomological Foundation, Bayer Environmental Science, the National Conference on Urban Entomology, the International Union for the Study of Social Insects, the Virginia Pest Management Association, and the Hispanic Scholarship Fund. I am also very grateful to Dow AgroSciences for their fellowship support through the GEM Fellowship Program.

Table of Contents

Introduction 1

Chapter One
Literature review: termites and their symbiotic gut microbiota 3

Chapter Two
The use of amplified rDNA restriction analysis (ARDRA) as a tool for studying the gut bacteria of termites 27

Chapter Three
Diversity of the gut microbiota of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) as described by 16S rRNA gene sequencing and amplified rDNA restriction analysis (ARDRA) 54

Chapter Four
Effects of food source on the gut microbiota of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) 80

Chapter Five
Analysis of the bacterial diversity between species of termites: evidences for coevolution 96

Vita 111

List of Tables

2.1 Estimations of the diversity of the bacteria in the termite gut using the Curtis and Chao1 estimators. 38

2.2 Indices of diversity for each of the samples PP-1, PP-2, PT-1, and BF-1. 38

2.3 Results of the two-sample test for binomial proportions. 39

2.4 Similarity/dissimilarity indices of termite gut bacterial ARDRA profiles for inter- and intracolony comparisons. 39

2.5 Assignment of clones from this study to known 16S rRNA bacterial sequences based on ARDRA profiles. The table matches the ARDRA profiles of clones from this study to digitally generated ARDRA profiles of known 16S rRNA gene sequences. All sequences with accession #'s beginning with "AB" were from *R. speratus*. Those beginning with "DQ" were from *R. flavipes*. 46

3.1 Assignment of clones from this study to known 16S rRNA bacterial sequences based on ARDRA profiles. The table matches the ARDRA profiles of clones from this study to digitally generated ARDRA profiles of known 16S rRNA gene sequences. 72

4.1 Data showing the aggression index (AG) for pairings of termites. The first three pairings show data for intracolony pairings of individuals fed on two different food sources (A&B). The last three show the AG of intercolony pairings. The control AG is the aggression index of intracolony pairings fed on the same food source (white pine). 89

4.2 Similarity/dissimilairty indices comparing the termite gut bacteria ARDRA profiles of three samples (PP-1Orig, PP1-Recr, and PP-1Post) fed on different food sources. 91

List of Figures

2.1 An example of ARDRA profiles. Each lane of the gel is a different digested clone and each band is a DNA fragment. Fragment sizes are determined using the standards found in lanes 1 and 10. 36

2.2 Rarefaction curves for ARDRA profiles of bacteria from PP-1, PP-2, BF-1, and PT-1. The error bars represent the 95% confidence level of the expected number of profiles for each sample size. 37

2.3 Unrooted dendograms constructed from the Bray-Curtis sample dissimilarity data (A) and the Dice similarity coefficient data (B). Dendograms were constructed using the neighbor-joining algorithm. The scale bar represents the percentage of dissimilarity (0.1=10%) 40

2.4 Taxonomic composition of termite gut bacterial ARDRA profiles taken from *R. flavipes*. The taxonomic assignments were accomplished using digitally digested 16S rRNA sequences from *R. speratus* (see Table 2.4). 43

3.1 Phylogenetic tree of 16S rRNA gene sequences of *R. flavipes* gut bacteria. The scale bar represents 0.05 substitutions per base position. Bootstrap values above 50 for 1000 resamplings (≥50% of the trees support the node) are shown for each node. These sequences correspond to positions 22–1400 in *E. coli.* Black triangles delineate samples sequenced in this study. 62

3.2 Rarefaction curve for 16S rRNA gene sequences. The error bars represent the 95% confidence interval of the expected number of ribotypes for each sample size. 64

3.3 Rarefaction curve for ARDRA profiles. The error bars represent the 95% confidence interval of the expected number of different profiles for each sample size. 70

3.4 Taxonomic proportions of the termite gut bacteria as measured using all of the ARDRA profiles (512 profiles), all of the ARDRA profiles except the "unknowns" (208 profiles), and 16S rRNA gene sequences from 42 samples. 71

4.1 Rarefaction curves of the gut bacterial ARDRA profiles for PP-1Recr, PP-1Post, and PP-1Orig. The error bars represent 95% confidence intervals for the expected number of profiles. 91

5.1 Neighbor-joining phylogenetic tree showing the relationships of 16S rRNA ribotypes of termite gut bacteria affiliated with the phylum *Bacteroidetes*. Only bootstrap values ≥50 for 1000 resamplings are shown. Only positions corresponding to positions 22-1400 in *E. coli* are used in the analysis. The scale bar represents 0.02 substitutions per base position. The labels for each sample are given as "accession #|ribotype taxonomy (corresponding termite genus)." Cluster names are shown in bold. Those ribotypes with known affiliation to symbiotic protozoa are labeled with an *. Those ribotypes from this study are labeled with a **. 104

5.2 Neighbor-joining phylogenetic tree showing the relationships of 16S rRNA ribotypes of termite gut bacteria affiliated with the phylum *Actinobacteria*. Only bootstrap values ≥50 for 1000 resamplings are shown. Only positions corresponding to positions 22-1400 in *E. coli* are used in the analysis. The scale bar represents 0.02 substitutions per base position. The labels for each sample are given as "accession #|ribotype taxonomy (corresponding termite genus)." Cluster names are shown to the right side in bold. Those ribotypes from this study are labeled with a *. 105

5.3 Neighbor-joining phylogenetic tree showing the relationships of 16S rRNA ribotypes of termite gut bacteria affiliated with the proposed phylum "*Endomicrobia*." Only bootstrap values ≥50 for 1000 resamplings are shown. Only positions corresponding to positions 22-1400 in *E. coli* are used in the analysis. The scale bar represents 0.005 substitutions per base position. The labels for each sample are given as "accession #|ribotype taxonomy (corresponding termite genus)." Cluster names are shown in bold. 106

5.4 Neighbor-joining phylogenetic tree showing the relationships of 16S rRNA ribotypes of termite gut bacteria affiliated with the phylum *Spirochaetes*. Only bootstrap values ≥50 for 1000 resamplings are shown. Only positions corresponding to positions 22-1400 in *E. coli* are used in the analysis. The scale bar represents 0.02 substitutions per base position. The labels for each sample are given as "accession #|ribotype taxonomy (corresponding termite genus)." Cluster names are shown in bold. 107

Introduction

Reticulitermes flavipes (Isoptera: Rhinotermitidae) the eastern subterranean termite, is an economically important termite species indigenous to North America. Within the gut of *R. flavipes* resides a diverse group of symbiotic microorganisms, including bacteria and protozoa. The bacteria and protozoa perform essential functions for *R. flavipes*, including cellulose digestion, nitrogen acquisition, and vitamin production.

Due to their specific physiological needs, the termite gut bacteria are very difficult to culture. Several culture independent methods have been developed, however, for studying the termite gut microbiota. The most common method in the literature is polymerase chain reaction (PCR)-based 16S rRNA gene sequencing. This method has high taxonomic resolving power but is labor intensive and expensive. As part of this study, I utilized 16S rRNA gene sequences to study the diversity of the gut bacteria in *R. flavipes* and included the sequences as part of a study investigating the coevolution of termites and their gut bacterial symbionts.

Because of the inherent limitations of 16S rRNA gene sequencing, I decided to assess the utility of fluorescent *in situ* hybridization (FISH) probe analysis in conjunction with flow cytometry for the study of the termite gut bacteria. Flow cytometry is a technique used to count cells suspended in a liquid. The suspended cells are labeled with fluorescent probes and passed individually through a very small cylindrical detecting chamber where they are subjected to laser excitation. A device within the detecting chamber counts the number of labeled and unlabeled cells that flow through the chamber. Following established protocols, I was not able to effectively use the methodology to

obtain useful data. The probes used were EUB338 (targets most Eubacteria), CF319a (targets the CFB bacterial group), and TG1-Pv-Rsa (targets "*Endomicrobia*" bacteria). I expected to find more cells labeled with EUB338 than with either CF319a or TG1-Pv-Rsa. For unknown reasons, however, the flow cytometer was unable to detect differences in population densities between samples labeled with EUB338 and those labeled with CF319a and TG1-Pv-Rsa. Thus I concluded that FISH probe analysis with flow cytometry would not be an effective technique for studying the gut bacterial community in *R. flavipes*.

As an alternative to FISH probe/flow cytometry analysis, I examined the usefulness of amplified rDNA restriction analysis (ARDRA). Compared to other molecular fingerprinting methods, ARDRA has higher taxonomic resolution, is reproducible, and does not require the additional equipment used by other fingerprinting techniques. Compared to 16S rRNA gene sequencing, ARDRA is considerably less expensive. This dissertation examined ARDRA as an alternative method to sequencing for investigating the diversity and ecology of the gut microbiota of *R. flavipes*. ARDRA was used to successfully characterize the gut bacterial diversity and to try to determine if the termite gut microbiota could be manipulated by changing the host termite's food source. I intend for this dissertation to be a springboard for further research into the diversity, function, and biotechnological importance of the termite gut microbiota.

Chapter One
Literature Review: Termites and Their Symbiotic Gut Microbiota

Termites are a moderately diverse group of hemimetabolous insects belonging to the order Isoptera. The order Isoptera is comprised of approximately 2,700 species (Zoological Society of London, 1995/1996) and distributed across all continents with the exception of Antarctica. The vast majority of species, however, are found in the tropics (Wood and Sands 1978). The United States is home to only about 45 species of termites (Potter 1997).

The termites, along with mantids (order: Mantodea) and cockroaches (order: Blatteria), form the most basal group of winged insects, sometimes referred to as the Dictyoptera (Deitz et al. 2003). The order Isoptera is further divided, depending on the source, into four to seven families (Noirot 1992, Kambhampati et al. 1996, Abe et al. 2000, Triplehorn and Johnson 2005). The families of termites are further classified as either higher or lower termites (Krishna and Weesner 1970). The lower termites are distinguished from the higher termites by a presence of symbiotic protozoa in their hindgut. Whereas the lower termites depend on the protozoa for cellulose digestion, the higher termites have developed other mechanisms for digesting cellulose without the help of protozoa (e.g. symbiotic fungi and higher endogenous cellulase production levels) (Breznak and Brune 1994).

Termites are eusocial insects. Eusociality is characterized by groups who possess three main characteristics: (1) cooperation in caring for brood, (2) reproductive division of labor, and (3) overlap of generations (de la Torre-Bueno 1989). The evolution of eusocial behavior theoretically followed a path from solitary individuals, to one or more

different levels of presocial groups, to finally eusocial colonies (Wilson 1971). Hamilton (1963, 1964, 1972) predicted that eusociality develops as the relatedness between altruist and beneficiary is higher than the ratio of costs to benefits. Hymenoptera have a haplo-diploid system for sex determination while termites are diplo-diploid (Crozier and Pamilo 1996). Female hymenopterans are more related to their siblings than to their own offspring. This type of relatedness leads to an increase in their inclusive fitness when raising female siblings and not their own offspring (Hartl and Brown 1970). Diploid termites lack this characteristic. The fact that termites are not haplo-diploid and have still developed eusociality has baffled scientists (Thorne 1997). Several theories have been proposed, however, trying to account for the evolution of eusociality in termites (Thorne 1997). Most agree that a variety of factors played important parts in the evolution of termite eusociality.

Termites are integral ecological components within their environments. The termite diet consists mainly of cellulose material, such as wood, leaf litter, grasses, roots, dung, fungi, and humus. Termites digest the cellulose into useable nutrients (Cleveland 1926). Thus termites perform an important ecological role by converting indigestible cellulose materials into insect biomass. The insect biomass is then available as a significant nutritional component of the ecosystem. In tropical areas, termite numbers can exceed 6,000 individuals per square meter, and their biomass can often be greater than mammalian herbivores (Lee and Wood 1971, Collins 1989).

Termites display complicated eusocial behavior. Multiple castes, such as reproductives, soldiers, workers, and nymphs, make up highly organized and complex colonies (Roison 2000). Each caste performs important functions for the colony. The

function of the reproductive caste includes both dispersal and reproduction. Winged reproductives, which are the main dispersal mechanism in termites, are termed “alates” (Thorne 1996). Upon dispersing from its natal colony, an alate pairs with an individual of the opposite sex, looses its wings, and founds a new colony. Depending on the species of termite, colonies can contain multiple male and female reproductives or only a single king and queen (Atkinson and Adams 1997, Myles 1999). The worker caste performs most of the foraging and colony maintenance. Some species have a strict worker caste, while other species demonstrate greater plasticity by allowing workers, depending on colony need, to molt into soldiers or reproductives (Roison 2000). Soldiers specialize in defending the colony. Specialized mandibles, larger heads, and aggressive defensive behaviors make the soldier adept at defending the colony from invaders. The specialized mandibles of soldiers make them unable to feed themselves, thus the soldiers rely heavily upon the workers for nourishment via trophallaxis (Roison 2000).

Trophallaxis is the process wherein individuals within the colony mutually exchange food and nutrients (Snyder 1948). Nestmates share both regurgitated and anally excreted food materials. One of the purposes of trophallaxis in termites is to replace the symbiotic microbiota in the hindgut of termites that have recently molted. When a termite molts, it loses the lining of the hindgut, which is composed of exoskeleton, and all of the contents of the hindgut, including the microbiota. Thus termites must reacquire the necessary gut symbionts to survive. The termites reacquire their symbiotic microbiota by participating in stomodeal and proctodeal trophallaxis (Snyder 1948).

Reticulitermes flavipes. Subterranean termites, specifically those in the family Rhinotermitidae, are some of the most important structural pests in the United States (Su and Scheffrahn 1990). Within Rhinotermitidae, *Reticulitermes flavipes*, *R. virginicus*, *R. hageni*, *R. hesperus*, and *Coptotermes formosanus*, an introduced species, form the core group of species responsible for the majority of termite damage. It has been estimated that termites cause billions of dollars in damage each year in the United States (Pinto 1981, Edwards and Mill 1986).

The eastern subterranean termite, *R. flavipes* (Kollar), is a lower termite species native to North America. It is most commonly found in the warm, southeastern portion of the United States (Su et al. 2001). *R. flavipes* was originally described in 1837 at Schönbrunn Palace in Vienna, Austria, where it had been transported in root stocks from its native habitat in the United States (Kollar 1837). Its current distribution, including areas where it has been introduced as an exotic pest, includes North America, Europe (Kollar 1837), South America (Austin et al. 2005), and the Bahamas (Scheffrahn et al. 1999).

New colonies of *R. flavipes* are usually founded by a single male and a single female alate after swarming from their natal colonies (Thorne 1998). *R. flavipes* alates usually swarm on warm, moist days in the spring or late winter (Potter 1997). Thousands of alates emerge simultaneously from a single colony. After pairing with a member of the opposite sex, the royal pair sheds their wings, locates a suitable nesting location, excavates a nuptial chamber, mates, and begins brood rearing. Colony growth is slow at first, but the new colony may eventually contain many hundreds of thousands of individuals (Thorne 1998).

The life cycle of *R. flavipes* is complex. Upon hatching, immatures (or nymphs that are often incorrectly termed "larvae" in the literature) develop through several undifferentiated instars (Thorne 1998). These white immatures are nutritionally dependent on their older nestmates. The immatures then develop into individuals that belong to one of several distinct morphological or developmental lines. As listed previously, these include the soldier, worker, and reproductive (sometimes termed "nymphal") lines (Thorne 1998). In *R. flavipes*, another type of morphotype, termed "pseudergates," includes non-reproductive individuals that revert from the reproductive line into worker-like individuals (Grassé and Noirot 1947, Thorne 1996). Pseudergates retain the brain and reproductive structures of reproductives, yet lack the wing-buds of their counterparts (Thorne 1996). In addition, pseudergates have the ability to differentiate into soldiers. Another distinct developmental characteristic of *R. flavipes* colonies is the existence of at least three different types of reproductives (Thorne 1998). These include the primary reproductives, usually the original founding king and queen, and two additional forms of secondary, or "neotenic," reproductives (Thorne 1998). The two forms of secondary reproductives are the "brachypterous" and "apterous" neotenics (Thorne 1998). Brachypterous neotenics originate from the reproductive line but do not fully develop into alates. Instead, they only develop wing-buds before becoming reproductively active within their own natal colony. The apterous neotenics are derived from the worker line (Thorne 1998). Similar to the brachypterous neotenics, they remain in the natal colony, yet they have no wing-buds. Since both types remain in the original colony, brachypterous and apterous nymphs are sometimes referred to as "supplemental

reproductives" (or "replacement reproductives" in the case of the death of a primary reproductive) (Thorne 1998).

The colony structure of *R. flavipes* is dynamic and diverse. Howard et al. (1982) meticulously dissected six *R. flavipes* colonies in Mississippi. These colonies ranged in size from 51,000 to 363,000 individuals, demonstrating that colonies are extremely variable in the number of termites they contain. In addition, colonies can either be localized within a small geographic area or expand their foraging territory away from the central nest for up to 79 m (Grace et al. 1989, Su et al. 1993, Forschler 1994). Su et al. (1993) suggested that termite colonies may forage within an area as large as 2,000 m^2.

R. flavipes colonies also demonstrate diversity in their breeding systems and population structures. Initially it was believed that all colonies of *R. flavipes* were genetically closed systems. However, Jenkins et al. (1999) produced mitochondrial DNA evidence for multiple maternal lineages in *R. flavipes* colonies. Jenkins et al. (1999) proposed that these data were evidence of colonies that were either founded by more than one queen or that colonies were open to intercolony fusion. Later studies that used molecular fingerprinting methods suggested that *R. flavipes* colonies range from simple family structures with monogamous, alate-derived parents, to complex, interconnected nests containing numerous, unrelated breeding neotenic reproductives (Thorne et al. 1999, Bulmer et al. 2001). More recent studies, however, have shown that a great majority of colonies of *R. flavipes* are simple families headed by outbred primary reproductives while a minority are colonies headed by inbred replacement reproductives (Vargo 2003, Fisher et al. 2004, DeHeer and Vargo 2004).

Termite gut symbiotic microbiota. Within the gut of higher and lower termites resides a wide variety of symbiotic microorganisms. Bacteria, protozoa, fungi, and archaeans are commonly found within the termite gut. Many of these microorganisms perform essential functions for the termite. In return, the termite gut environment provides the microorganisms with an appropriate physiological environment and a steady supply of nutrients.

Lespes (1856) was the first person to describe the presence of microorganisms in the termite gut. This description was later supplemented by more detailed studies of *R. flavipes* (Leidy 1877, 1881). Leidy (1877, 1881) described the presence of spirochete bacteria and eight new species of protozoa in the hindgut, in particular the common archaeoprotists, *Trychonympha agilis*, *Pyrsonympha verteus*, and *Dinenympha gracilis*. Leidy (1877, 1881) also determined that the bacteria and protozoa were not harmful to the termite and that they depended entirely upon their termite hosts. He incorrectly concluded, however, that the bacteria and protozoa were unnecessary guests in the termite gut. It was Cleveland (1926) who finally recognized that termites were dependent on their gut symbionts (see also Eutick et al. 1978b).

Since the initial studies by Lespes (1856) and Leidy (1877, 1881), much work has been done to elucidate the diversity, ecology, and function of the microorganisms in the termite gut. It is now understood that the gut microbiota consists of a diverse array of bacterial and protozoan species. Yamin (1979) did a survey of the protozoan species that had been discovered in the gut of the lower termites and three species of the cockroach genus *Cryptocercus*. Yamin (1979) found that at least 434 different species of protozoa had been described. In *R. flavipes*, at least 12 species of protozoa have been described as

inhabiting the termite gut (Yamin 1979). These species of protozoa were divided into two main groups: the Parabasalia (including the hypermastigid and trichomonad flagellates) and the Oxymonadida (Yamin 1979).

The bacteria in the termite gut are far more diverse than the protozoa. In *R. speratus*, a lower termite species closely related to *R. flavipes*, Hongoh et al. (2003) found 268 different bacterial phylotypes (equivalents to species) out of a sample of 1,344 16S rRNA gene clones. Hongoh et al. (2003) concluded that the gut of *R. speratus* may harbor as many as 700 different phylotypes. The phylotypes found so far can be classified into many different bacterial phyla, including *Spirochaetes*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, the recently proposed "*Endomicrobia*" (Stingl et al. 2005), *Planctomycetes*, *Verrucomicrobia*, *Cyanobacteria*, *Acidobacteria*, and a number of other rare phyla (Hongoh et al. 2003). Breznak and Pankratz (1977) conducted a microscopic survey of the gut symbionts in *R. flavipes* and *C. formosanus*. They found that the gut of *R. flavipes* likewise consisted of morphologically diverse bacteria. A culture-based study of the gut symbionts in *R. flavipes* also yielded a diversity of anaerobic bacteria, including specimens of *Bacteroides*, Enterobacteriaceae, and *Streptococcus* (Schultz and Breznak 1978). Many studies have characterized the bacteria in a variety of other termite species (Eutick et al. 1978a, Paster et al. 1996, Ohkuma and Kudo 1998, Iida et al. 2000, Wenzel et al. 2002, Schmitt-Wagner et al. 2003; Hongoh et al. 2005a, 2005b; Shinzato et al. 2005, Yang et al. 2005).

The microorganisms in the termite gut form a dense population unequally distributed throughout the gut. Cook and Gold (1998) found that the protozoa in the hindgut of *R. flavipes* exceeded 20,000 individuals per hindgut, which has a volume of

only ~0.7 µl (Tholen and Brune 2000). The high concentration of protozoa in the gut of lower termites has been determined to account for up to half of the fresh weight of their host termites (Katzon and Kirby 1939). The protozoa, however, are not evenly distributed throughout the gut, but are mainly confined to the hindgut and absent in the fore- and midgut (Breznak and Brune 1994).

The bacteria are also dense and unequally distributed. Krasil'nikov and Satdykov (1969) estimated that in the termite *Anacanthotermes ahngerianus*, as many as 7.7×10^7 bacteria inhabited the gut. Nakajima et al. (2005) studied the gut of *R. speratus* and also discovered that the bacterial communities were densely populated, but they found that those bacteria associated with the gut fluid and the gut wall were phylogenetically different. *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* were the dominant species on the gut wall, but *Spirochaetes* and "*Endomicrobia*" were more dominant in the gut fluid fractions. Recently it has been discovered that some bacterial species, such as those in "*Endomicrobia*" and the genus *Treponema*, are ecto- and endosymbionts of the gut protozoa (Noda et al. 2003, Stingl et al. 2005). In addition, major differences in the density and distribution of the bacteria exist between the fore-, mid-, and hindguts of termites (Krasil'nikov and Satdykov 1969, Breznak and Brune 1994).

The symbionts in the termite gut perform many important functions. Gut symbionts have been found to perform a vital role in cellulose digestion in the lower termites. Protozoa in the hindgut produce cellulases that break down cellulose into acetate, which is in turn absorbed by the termite as its main energy and carbon source (Honigberg 1969, Breznak and Switzer 1986, Leadbetter et al. 1999). In addition to

acetate, the bacteria and protozoa produce small amounts of other volatile fatty acids, such as propionate and butyrate (Odelson and Breznak 1983).

The gut bacteria of both higher and lower termites have also been implicated in nitrogen acquisition, an important function considering termites have nitrogen poor diets. Several authors have shown that nitrogen-fixing bacteria inhabit the termite gut (Benemann 1973, Breznak et al. 1973, French et al. 1976). The rate of nitrogen-fixation varies depending on termite species (Prestwich et al. 1980, Breznak 1984, Bentley 1987, Waller et al. 1989), termite caste (Prestwich et al. 1980, Hewitt et al. 1987), termite size (Waller et al. 1989), and food sources (Breznak et al. 1973). The newly fixed nitrogen is incorporated into the termite's tissues, excrement, and secretion products (Bentley 1984). In addition to nitrogen-fixation, the gut bacteria recycle uric acid, providing the termite with another source of nitrogen (Potrikus and Breznak 1980, 1981).

It has been suggested that another possible function of the termite gut microorganisms may include a role in intra- and intercolony recognition. Adams (1991) made several interesting statements in his discussion of termite interaction:

> "Workers of (*Microcerotermes*) *arboreus* recognized unfamiliar relatives and distinguished degrees of similarity among unfamiliar kin. These results demonstrate that there is a strong inherited component to the cues used in nest-mate recognition…
>
> …the possibility of nongenetic inheritance cannot be ruled out. Odors may be carried from the source nest by alate reproductives and transferred directly to their offspring…Symbiotic bacteria used in digestion of cellulose are also transferred from parent to offspring, but there is no evidence that these affect surface pheromones."

The three key points in Adam's (1991) discussion were (1) that nestmate recognition cues are inherited, (2) that non-genetic inheritance needs to be considered, and (3) that symbiotic bacteria are a nongenetically inherited factor.

Alates, by way of trophallaxis, inoculate their newly formed colonies with symbiotic microorganisms, providing a nongenetically inherited component to their offspring (Snyder 1948). As the colony grows, the job of faunating the newly hatched termites is performed by the workers. In addition, the workers constantly participate in trophallaxis amongst themselves (Snyder 1948). Thus, due to the continual exchange of digestive fluids within a colony, all members of a colony, in theory, should have a similar gut microbiota originally inherited from the founding alates.

As previously mentioned, Adams (1991) stated that no evidence existed that the termite microbiota performs any role in constructing nestmate recognition surface pheromones. Matsuura (2001) found, however, that intestinal bacteria in *R. speratus* mediated nestmate recognition. In his study, Matsuura (2001) used antibiotics and bacterial extracts to show that bacteria influence the degree of aggression demonstrated between and within colonies of *R. speratus*. The chemicals given off by bacteria in the gut and excreted in termite feces might be the chemical odors termites use in nestmate recognition (Matsuura 2001).

The findings of Matsuura (2001) are particularly interesting in light of a study by Shelton and Grace (1997), which concluded that an unknown environmental influence might perform an important role in a "multiple component system for intercolony kin recognition" in *C. formosanus.* Shelton and Grace (1997) found that several pairings of unrelated colonies demonstrated that no antagonism existed between "exogenously similar, but genetically different, laboratory colonies." Since nestmate recognition is not correlated with genetic relatedness, doubt is cast on the hypothesis that the termite's DNA is the heritable factor involved in termite nestmate recognition.

It is possible that Matsuura's bacteria are the environmental influence suggested by Shelton and Grace (1997) and the heritable factor described by Adams (1991) that regulates nestmate recognition. This idea becomes more compelling as one discovers that the gut microbiota of termites are heavily influenced by environmental factors such as temperature (Belitz and Waller 1998), oxygen level (Tholen et al. 1997), and food source (Mannesmann 1972, Cook and Gold 2000). If it can then be assumed that if bacterially derived, endogenous chemicals are the cues termites use in nestmate recognition, then changes to the environment will change the gut bacterial microbiota and in turn the nestmate recognition cues. Thus bacteria would tie together seemingly contradictory studies implicating either environmental (Shelton and Grace 1997) or heritable factors (Adams 1991) as important in termite nestmate recognition.

Research Justification. Over the past two decades, studies have demonstrated that most (>99%) of the bacteria present in environmental samples, including termite guts, cannot be cultivated in the laboratory (Sharma et al. 2005). Recent research has revealed that most of these unculturable bacteria are new species and belong to undescribed classes and divisions in the domains Eubacteria and Archaea (Hugenholtz et al. 1998). Thus most of the ecological impacts and biotechnological applications of this unculturable majority remain unseen (Kellenberger 2001).

Research on newly discovered unculturable termite gut microorganisms has already yielded discoveries that may have important ecological impacts and biotechnological applications. Speaking at the Institute of Physics 2005 conference in Warwick, United Kingdom, Nobel laureate for physics Steven Chu suggested that the termite gut may hold an important key in solving the world's energy problems (Chu

2005). Chu argued that evolution has produced in termites a carbon-neutral method for turning cellulose into ethanol. Some of the bacteria in the termite gut efficiently take low-grade fuel sources, such as plant matter, and convert them into fuel that releases no more carbon than it took to produce it.

Another use of the termite gut microbiota has been proposed by Philip Hugenholtz and Jared Leadbetter at the Joint Genome Institute in Walnut Creek, California (Platoni 2005). Hugenholtz has proposed that the termite gut bacteria are promising candidates for hydrogen fuel production (Platoni 2005). Termite gut bacteria produce enzymes that efficiently generate hydrogen as a cellulose digestion byproduct (Taguchi et al. 1992, Ebert and Brune 1997, Sugimoto et al. 1998, Schmitt-Wagner and Brune 1999). Splicing the genes responsible for hydrogen production into cultivable bacteria may result in an efficient hydrogen fuel source. If the genes possessed by the gut microbiota of termites may hold the key to the world's energy problems, it begs the question: "If there are thousands of undescribed termite gut microorganisms with an innumerable amount of undescribed genes, what other ecological and biotechnological discoveries await those who study the termite-symbiont system?"

Another practical use of the termite gut microbiota involves possible new termite management techniques. The hypothesis that bacteria perform an important role in termite nestmate recognition has termite management implications. Understanding how termites distinguish one another can be used as a potential termite management strategy. Thorne and Haverty (1991) outlined how antagonistic behaviors in termites could be used to control termites. They suggested that control approaches could be designed wherein nestmate recognition stimuli within a termite colony are masked or obstructed. The

outcome would be a "civil war" resulting in colony death. In addition, the authors proposed that building materials could be impregnated with substances that elicit antagonistic behaviors, thus preventing termite colonization.

It has also been proposed that termites might be controlled by targeting their gut microbiota via a technique termed "paratransgenesis" (Peloquin et al. 2000, Husseneder et al. 2005, Husseneder and Grace 2005). Paratransgenesis describes a process wherein bacteria are genetically modified to act as "shuttles" to deliver and express foreign genes within a target host. In termites, the foreign gene may express a toxin deadly to termites or to their gut symbionts. The bacteria used in a study by Husseneder et al. (2005) do not naturally occur in large numbers in termites and had difficulty persisting in the termite gut. Therefore, Husseneder and Grace (2005) genetically modified an indigenous bacterial species, *Enterobacter cloacae*, and demonstrated its ability to act as a persistent shuttle system in *C. formosanus*. Thus, the termite-symbiont relationship was exploited as a possible termite management technique.

One of the selective pressures that led to the development of eusocial behavior in termites may be found in their gut microbiota. Lower termites are dependent on their microbiota for their ability to digest cellulose while the microorganisms are dependent on the termites for harborage and dispersal. The presence of symbiotic microorganisms may have predisposed termites to gregariousness due to the fact that microbiota must be passed from nestmate to nestmate via trophallaxis (Thorne 1997). This need for nestmate contact would account for one of the characteristics of eusociality, overlapping generations. Cooperative brood care, another characteristic of eusociality, is also the natural result of gut symbiont exchange between nestmates. Newly hatched nymphs

require intimate association with mature termites in order to receive their initial inoculation of microorganisms. In addition, the relationship between mature and immature termites must continue throughout nymphal development because juveniles must be refaunated after each molt (Thorne 1997).

The microbiota may also enter the eusocial evolutionary picture due to their potential role in termite nestmate recognition. Wilson (1971) explains that the ability to recognize nestmates as nestmates "form(s) much of the central repertory of social life." If such a mechanism were not in place, parasitism by other species or foreign termite colonies would eventually break down the colony's social boundaries. Therefore, social insects must develop means to distinguish aliens from nestmates in order to protect their resources. If gut bacteria are producers of colony specific nestmate recognition odors, then bacteria would be the "glue" that holds the colony together. Therefore, the gut microorganisms may have performed a very important role in the evolution of eusocial behavior in diploid termites. Thus studying the relationship between the termites and their gut microbiota could answer important questions about termite eusocial evolution.

Summary. Termites are a diverse group of insects that perform an important ecological role in their environments. The gut of the so-called lower termites includes a diverse array of symbiotic microorganisms. The microorganisms perform several important functions for their termite hosts including cellulose digestion and nitrogen aquisistion. Recent findings indicate that the gut microbiota of termites harbors several species of economically and ecologically important symbionts. Further studies of these important protozoa and bacteria are needed to elucidate their ecological role and explore their possible biotechnological uses.

References Cited

Abe, T., D. E. Bignell, and M. Higashi (eds.) 2000. Termites: evolution, sociality, symbiosis, ecology. Kluwer Academic Publishers, Dordrecht, Netherlands.

Adams, E. S. 1991. Nest-mate recognition based on heritable odors in the termite *Microtermes arboreus*. Proc. Natl. Acad. Sci. 88: 2031-2034.

Atkinson, L., and E. S. Adams. 1997. The origins and relatedness of multiple reproductives in colonies of the termite *Nasutitermes corniger*. Proc. R. Soc. Lond. B 264: 1131-1136.

Austin, J. W., A. L. Szalanski, R. H. Scheffrahn, M. T. Messenger, S. Dronnet, and A.–G. Bagnères. 2005. Genetic evidence for the synonymy of two *Reticulitermes* species *Reticulitermes flavipes* (Kollar) and *Reticulitermes santonensis* (Feytaud): Ann. Entomol. Soc. Am. 98: 395–401.

Belitz, L. A., and D. A. Waller. 1998. Effect of temperature and termite starvation on phagocytosis by protozoan symbionts of the Eastern subterranean termite *Reticulitermes flavipes*. Microbial Ecol. 36: 175-180.

Benemann, J. R. 1973. Nitrogen fixation in termites. Science 181: 164-165.

Bentley, B. L. 1987. Nitrogen fixation by *Nasutitermes* and *Velocitermes* in Venezuela, p. 365. *In* J. Eder and H. Remhold (eds.), Chemistry and biology of social insects, Verlag J. Peperny, Munich, Germany.

Breznak, J.A., W. J. Brill, J. W. Mertins, and H. C. Coppel. 1973. Nitrogen fixation in termites. Nature 244: 577-580.

Breznak, J. A., and A. Brune. 1994. Role of microorganisms in the digestion of lignocellulose by termites. Annu. Rev. Entomol. 39: 453-487.

Breznak, J. A., and H. S. Pankratz. 1977. In situ morphology of the gut microbiota of wood-eating termites [*Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki]. Appl. Environ. Microbiol. 33: 406-426.

Breznak, J. A. 1984. Biochemical aspects of symbiosis between termites and their intestinal microbiota, pp. 173-203. *In* J. M. Anderson, A. D. M. Rayner, and D.W.H. Walton (eds.), Invertebrate-microbial interactions, Cambridge Univ. Press, Cambridge, U.K.

Breznak, J. A., and J. M. Switzer. 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl. Environ. Microbiol. 52: 623–630.

Bulmer, M. S., E. S. Adams, and J. F. A. Traniello. 2001. Variation in colony structure in the subterranean termite *Reticulitermes flavipes*. Behav. Ecol. Sociobiol. 49: 236-243.

Chu, S. 2005. Biology as a solution to physics problems. Plenary speech, Institute of Physics Conference: Physics 2005, 14 April 2005, Warwick, UK.

Cleveland, L. R. 1926. Symbiosis among animals with special reference to termites and their intestinal flagellates. Quart. Rev. Biol. 1: 51-64.

Collins, N. M. 1989. Termites, pp. 455-472. *In* H. Lieth and M. J. A. Werger (eds.), Ecosystems of the world 14B: Tropical rain forest systems. Biogeographical and ecological studies, vol. 2, Elsevier, Amsterdam, Netherlands.

Cook, T. J., and R. E. Gold. 1998. Organization of the symbiotic flagellate community in three castes of the eastern subterranean termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Sociobiology 31: 25-39.

Cook, T. J., and R. E. Gold. 2000. Effects of different cellulose sources on the structure of the hindgut flagellate community in *Reticulitermes virginicus* (Isoptera: Rhinotermitidae). Sociobiology 35: 119-130.

Crozier, R. H., and P. Pamilo. 1996. Evolution of social insect colonies. Oxford University Press, Oxford, UK.

de la Torre-Bueno, J. R. 1989. The Torre-Bueno glossary of entomology, rev. ed. New York Entomological Society, New York, NY.

DeHeer, C. J., and E. L. Vargo. 2004. Colony genetic organization and colony fusion in the termite *Reticulitermes flavipes* as revealed by foraging patterns over time and space. Mol. Ecol. 13: 431-441.

Deitz, L. L., C. Nalepa, and K.–D. Klass. 2003. Phylogeny of the Dictyoptera re-examined (Insecta). Entomol. Abhandl. 61: 69-91.

Ebert, A., and A. Brune. 1997. Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite *Reticulitermes flavipes* (Kollar). Appl. Environ. Microbiol. 63: 4039-4046.

Edwards, R., and A. E. Mill. 1986. Termites in buildings: their biology and control. Rentokill Limited, W. Sussex, UK.

Eutick, M. L., R. W. O'Brian, and M. Slaytor, 1978a. Bacteria from the gut of Australian termites. Appl. Environ. Microbiol. 35: 823-828.

Eutick, M. L., P. Veivers, R. W. O'Brien, and M. Slaytor. 1978b. Dependence of the higher termite, *Nasutitermes exitiosus* and the lower termite, *Coptotermes lacteus* on their gut flora. J. Insect Physiol. 24: 363-368.

Fisher, M. L., R. E. Gold, E. L. Vargo, and A. I. Cognato. 2004. Behavioral and genetic analysis of colony fusion in *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Sociobiology 44: 565-576.

Forschler, B. T. 1994. Fluorescent spray paint as a topical marker on subterranean termites (Isoptera: Rhinotermitidae). Sociobiology 24: 27-38.

French, J. R. J., G. L. Turner, and J. F. Bradbury. 1976. Nitrogen fixation by bacteria from the hindgut of termites. J. Gen. Microbiol. 95: 202-206.

Grace, J. K., A. Abdallay, and K. R. Farr. 1989. Eastern subterranean termite (Isoptera: Rhinotermitidae) foraging territories and populations in Toronto. Can. Entomol. 121: 551-556.

Grassé, P. P., and C. Noirot. 1947. Le polymorphisme sociale du termite à cou jaune (*Calotermes flavicollis* F.). Les faux-ouvriers ou pseudergates et les mues régressives. C. R. Acad. Sci. 224: 219-221.

Hamilton, W. D. 1963. The evolution of altruistic behavior. Am. Nat. 97: 354-356.

Hamilton, W. D. 1964. The genetical evolution of social behavior. J. Theor. Biol. 7: 1-52.

Hamilton, W. D. 1972. Altruism and related phenomena, mainly in social insects. Ann. Rev. Ecol. Syst. 3: 193-232.

Hartl, D. L., and S. W. Brown. 1970. The origin of male haploid genetic systems and their expected sex ratio. Theor. Pop. Biol. 1: 165-190.

Hewitt, P. H., M. C. Van der Westhuizen, T. C. De K. Van der Linde, and R. A. Adam. 1987. Acetylene reduction by the harvester termite *Hodotermes mossambicus* (Hagen). J. Entomol. Soc. S. Africa 50: 513-520.

Hongoh, Y., M. Ohkuma, and T. Kudo. 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol. Ecol. 44:231-242.

Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, and T. Kudo. 2005a. Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl. Environ. Microbiol. 71: 6590-6599.

Hongoh, Y., L. Ekpornprasit, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, N. Noparatnaraporn, and T. Kudo. 2005b. Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. Mol. Ecol. 15: 505-516.

Honigberg, B. M. 1969. Protozoa associated with termites and their role in digestion, pp. 1-36. *In* K. Krishna and F. M. Weesner (eds.), Biology of termites, vol. 2, Academic Press, New York, NY.

Howard, R. W., S. C. Jones, J. K. Mauldin, and R. H. Beal. 1982. Abundance, distribution, and colony size estimates for *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in southern Mississippi. Environ. Entomol. 11: 1290-1293.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180: 4765-4774.

Husseneder, C., J. K. Grace, and D. E. Oishi. 2005. Use of genetically engineered *Escherichia coli* to monitor ingestion, loss, and transfer of bacteria in termites. Curr. Microbiol. 50:119-123.

Husseneder, C., and J. K. Grace. 2005. Genetically engineered termite gut bacteria deliver and spread foreign genes in termite colonies. Appl. Microbiol. Biotechnol. 68: 360-367.

Iida, T., M. Ohkuma, K. Ohtoko, and T. Kudo. 2000. Symbiotic spirochetes in the termite hindgut: phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol. Ecol. 34: 17-26.

Kambhampati, S., K. M. Kjer, and B. L. Thorne. 1996. Phylogenetic relationship among termite families based on DNA sequence of mitochondrial 16S ribosomal RNA gene. Insect Mol. Biol. 5: 229-238.

Katzin, L. I., and H. Kirby. 1939. The relative weight of termites and their protozoa. J. Parasitol. 25: 444-445.

Kellenberger, E. 2001. Exploring the unknown: the silent revolution of microbiology. EMBO Rep. 2: 5-7.

Kollar, V. 1837. Naturgeschichte der schädlichen Insekten. Verb. Landwirtsch. Ges. Wien (N.S.): 411-413.

Krasil'nikov, N. A., and S. I. Satdykov. 1969. Estimation of the total bacteria in the intestines of termites. Mikrobiologiya 38: 346-350.

Krishna, K., and F. M. Weesner (eds.) 1970. Biology of termites. Academic Publishers, New York, NY.

Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283: 686-689.

Lee, K. E., and T. G. Wood. 1971. Termites and soils. Academic Press, New York, NY.

Leidy, J, 1877. On intestinal parasites of *Termes flavipes*. Proc. Natl. Acad. Sci. Phila. (ser. 2) 8: 146-149.

Leidy, J. 1881. Parasites of the termites. J. Acad. Nat. Sci. Phila. 3: 425-447.

Lespes, C. 1856. Recherches sur l'organisation et les moeurs du *Termite lucifuge*. Ann. Sci. Nat., Zool. Ser. 4 227: pg. unknown.

Mannesmann, R. 1972. Relationship between different wood species as a termite food source and the reproduction rate of termite symbionts. Z. Ang. Ent. 72: 116-128.

Matsuura, K. 2001. Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos 92: 20-26.

Myles, T. G. 1999. Review of secondary reproduction in termites (Insecta: Isoptera) with comments on its role in termite ecology and social evolution. Sociobiology 33: 1-91.

Nakajima, H., Y. Hongoh, R. Usami, T. Kudo, and M. Ohkuma. 2005. Spatial distribution of bacterial phylotypes in the gut of the termite *Reticulitermes speratus* and the bacterial community colonizing the gut epithelium. FEMS Microbiol. Ecol. 54: 247-255.

Noda, S., M. Ohkuma, A. Yamada, Y. Hongoh, and T. Kudo. 2003. Phylogenetic position and in situ identification of ectosymbiotic spirochetes on protists in the termite gut. Appl. Environ. Microbiol. 69: 625-633.

Noirot, C. 1992. From wood- to humus-feeding: an important trend in termite evolution, pp. 107-119. *In* J. Billen (ed.), Biology and evolution of social insects, Leuven University Press, Leuven, Belgium.

Odelson, D. A., and J. A. Breznak. 1983. Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl. Environ. Microbiol. 45: 1602-1613.

Ohkuma, M., and T. Kudo. 1998. Phylogenetic analysis of the intestinal microflora of the termite *Cryptotermes domesticus*. FEMS Microbiol. Letters 164: 389-395.

Paster, B. J., F. E. Dewhirst, S. M. Cooke, V. Fussing, L. K. Poulsen, and J. A. Breznak. 1996. Phylogeny of not-yet-cultured spirochetes from termite guts. Appl. Environ. Microbiol. 62: 347-352.

Peloquin, J. J., L. V. Kuzina, C. R. Lauzon, and T. A. Miller. 2000. Transformation of internal extracellular bacteria isolated from *Rhagoletis completa* Cresson gut with enhanced green fluorescent protein. Curr. Microbiol. 40:367-371.

Pinto, L. J. 1981. The structural pest control industry: description and impact on the nation. National Pest Control Association, Vienna, VA.

Platoni, K. 2005. Bug juice: could termite guts hold the key to the world's energy problems? Don't laugh. East Bay Express, 7 Sept 2005.

Potrikus, C. J, and J. A. Breznak. 1980. Uric acid degrading bacteria in guts of termites (*Reticulitermes flavipes* [Kollar]). Appl. Environ. Microbiol. 40: 117-124.

Potrikus, C. J., and J. A. Breznak. 1981. Gut bacteria recycle uric acid nitrogen in termites: a strategy for nutrient conservation. Proc. Nat. Acad. Sci. USA 78: 4601-4605.

Potter, M. F. 1997. Termites, pp. 233-334. *In* S. A. Hedges and D. Moreland (eds.), Handbook of Pest Control. Mallis Handbook & Technical Training Company, Cleveland, OH.

Prestwich, G. D., and B. L. Bentley, 1981. Nitrogen fixation in intact colonies of the termite *Nasutitermes corniger*. Oecologia 49: 249-251.

Roison, Y. 2000. Diversity and evolution of caste patterns. *In* T. Abe, D. E. Bignell, and M. Higashi (eds.) Termites: evolution, sociality, symbiosis, and ecology. Kluwer Academic Publishers, Dordrecht, Netherlands.

Scheffrahn, R. H., J. A. Chase, J. R. Mangold, J. Krecek, and N.-Y. Su. 1999. First record of *Reticulitermes* (Isoptera: Rhinotermitidae) from the West Indies: *R. flavipes* on Grand Bahama Island. Fla. Entomol. 82: 480–482.

Schmitt-Wagner, D., and A. Brune. 1999. Hydrogen profiles and localization of methanogenic activities in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.). Appl. Environ. Microbiol. 65: 4490-4496.

Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune. 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). Appl. Environ. Microbiol. 69: 6018-6024.

Schultz, J. E., and J. A. Breznak. 1978. Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. Appl. Environ. Entomol. 35: 930-936.

Sharma, R., R. Ranjan, R. K. Kapardar, and A. Grover. 2005. 'Unculturable' bacterial diversity: an untapped resource. Curr. Sci. 89: 72-77.

Shelton, T. G., and J. K. Grace. 1997. Suggestion of an environmental influence on intercolony agonism of Formosan subterranean termites (Isoptera: Rhinotermitidae). Environ. Entomol. 26: 632-637.

Shinzato, N., M. Muramatsu, T. Matsui, and Y. Watanabe. 2005. Molecular phylogenetic diversity of the bacterial community in the gut of the termite *Coptotermes formosanus*. Biosci. Biotechnol. Biochem. 69: 1145-1155.

Snyder, T. E. 1948. Our enemy the termite. Comstock Publishing Company, Ithaca, NY.

Stingl, U., R. Radek, H. Yang, and A. Brune. 2005. "Endomicrobia": cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. Appl. Environ. Microbiol. 71: 1473-1479.

Su, N.–Y., and R. H. Scheffrahn. 1990. Economically important termites in the United States and their control. Sociobiology 17: 17-94.

Su, N.–Y., P. M. Ban, and R. H. Scheffrahn. 1993. Foraging populations and territories of the eastern subterranean termite (Isoptera: Rhinotermitidae) in southeastern Florida. Environ. Entomol. 22: 1113-1117.

Su, N.–Y., R. H. Scheffrahn, and B. J. Cabrera. 2001. Native subterranean termites: *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), *Reticulitermes hageni* Banks (Insecta: Isoptera: Rhinotermitidae). UF/IFAS, Ft. Lauderdale, FL. #EENY-212.

Sugimoto, A., T. Inoue, I. Tayasu, L. Miller, S. Takeichi, and T. Abe. 1998. Methane and hydrogen production in a termite-symbiont system. Ecol. Res. 13: 241-257.

Taguchi, F., J. D. Chang, S. Takiguchi, and M. Morimoto. 1992. Efficient hydrogen production from starch by a bacterium isolated from termites. J. Ferment. Bioeng. 73: 244-245.

Tholen, A., B. Schink, and A. Brune. 1997. The gut microflora of *Reticulitermes flavipes*, its relation to oxygen, and evidence for oxygen-dependent acetogenesis by the most abundant *Enterococcus* sp. FEMS Microbiol. Ecol. 24: 137-149.

Tholen, A. and A. Brune. 2000. Impact of oxygen on metabolic fluxes and in situ rates of reductive acetogenesis in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. Environ. Microbiol. 2: 436-449.

Thorne, B. L., and M. I. Haverty. 1991. An assessment of the potential uses of agonistic behaviors in termite control. *In* Proceedings of the Symposium on Current Research on Wood-destroying Organisms and Future Prospects for Protecting Wood in Use, 13 Sept 1989, Bend, Oregon.

Thorne, B. L. 1996. Termite terminology. Sociobiology 28: 253-263.

Thorne, B. L. 1997. Evolution of eusociality in termites. Annu. Rev. Ecol. Syst. 28: 27-54.

Thorne, B. L. 1998. Biology of the subterranean termites of the genus *Reticulitermes*. *In* NPCA Research Report on Subterranean Termites. National Pest Control Association, Dunn Loring, VA.

Thorne, B. L., J. F. A. Traniello, E. S. Adams., and M. Bulmer. 1999. Reproductive dynamics and colony structure of subterranean termites of the genus *Reticulitermes* (Isoptera: Rhinotermitidae): a review of the evidence from behavioral, ecological, and genetic studies. Ethol. Ecol. Evol. 11: 149-169.

Triplehorn, C. A., and N. F. Johnson. 2005. Borrer and DeLong's introduction to the study of insects. Thomson Brooks/Cole, Belmont, CA.

Vargo, E. L. 2003. Hierarchical analysis of colony and population genetic structure of the eastern subterranean termite, *Reticulitermes flavipes*, using two classes of molecular markers. Evolution 57: 2805-2818.

Waller, D. A., G. A. Breitenbeck, and J. P. La Fage. 1989. Variation in acetylene reduction by *Coptotermes formosanus* (Isoptera: Rhinotermitidae) related to colony source and termite size. Sociobiology 16: 191-196.

Wenzel, M., I. Schönig, M. Berchtold, P. Kämpfer, and H. König. 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. J. Appl. Microbiol. 92: 32-40.

Wilson, E.O. 1971. The insect societies. Harvard University Press, Cambridge, MA.

Wood, T. G., and W. A. Sands. 1978. The role of termites in ecosystems, pp. 245-292. *In* M. V. Brian (ed.), Production ecology of ants and termites, Cambridge University Press, Cambridge, MA.

Yamin, M. A. 1979. Flagellates of the orders Trichomonadida Kirby, Oxymonadida Grassé, and Hypermastigida Grassi & Foà reported from lower termites (Isoptera families Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae, and Serritermitidae) and from the wood-feeding roach *Cryptocercus* (Dictyoptera:Cryptocercidae). Sociobiology 4:1-119.

Yang, H., D. Schmitt-Wagner, U. Stingl, and A. Brune. 2005. Niche heterogeneity determines bacterial community structure in the termite gut (*Reticulitermes santonensis*). Environ. Microbiol. 7: 916-932.

Zoological Society of London. 1995/1996. Zoological record. Zoological Society of London, London, UK.

Chapter Two
The Use of Amplified rDNA Restriction Analysis (ARDRA) as a Tool for Studying the Gut Bacteria of Termites

Introduction

Termites, especially those in the family Rhinotermitidae, are considered one of the most economically important structural pests in the United States (Su and Scheffrahn 1990). It has been estimated that termites cause billions of dollars in damage each year in the United States alone (Pinto 1981, Edwards and Mills 1986). Thus the biology, ecology, evolution, and behavior of termites have been the focus of many recent studies.

The gut of lower termites harbors a diverse community of symbiotic bacteria and protozoa. These symbionts perform significant physiological functions for the termite and in return are provided with environmental protection and a constant supply of nutrients. The gut microorganisms have been implicated in nitrogen-fixation (Benemann 1973, Breznak et al. 1973, French et al. 1976) and nitrogen recycling (Potrikus and Breznak 1980, 1981), important roles considering the termite's nitrogen poor diet. In the lower termites, the gut symbionts have also been found to perform a vital task in cellulose digestion. Microorganisms in the hindgut produce cellulases that break down cellulose into acetate, the termite's main energy and carbon source (Honigberg 1969, Breznak and Switzer 1986, Leadbetter et al. 1999).

The bacteria in the gut of the lower termites may provide a rich reservoir of ecologically and biotechnologically useful genes, biochemical pathways, and model systems. Because of their strict physiological requirements, however, most bacteria from the termite gut are not cultivable in the laboratory (Sharma et al. 2005). Recent research has revealed that many of these uncultivable bacteria are undescribed species belonging

to new phylogenetic clades within Eubacteria and Archaea (Hugenholtz et al. 1998, Hongoh et al. 2005a). Thus the ecological impacts and biotechnological applications of this unculturable majority remain relatively unexplored (Kellenberger 2001). If there are thousands of undescribed termite gut microorganisms and each undescribed species contains thousands of undescribed genes, what other ecological and biotechnological discoveries await those who study the termite-symbiont system?

Molecular tools have recently become available to researchers desiring to study the diversity and community dynamics of the unculturable termite gut microorganisms. Debate continues, however, with regard to the most appropriate molecular technique. Considerations must be made of the costs (including time commitments, required equipment, etc.), reproducibility, necessary operational skills, and the existence of databases against which data comparisons can be made. Different techniques may sample the gut community differently and thus provide different types of information (Hillis et al. 1996).

One of the molecular techniques that has been extensively used to study the termite gut microorganisms is 16S rRNA gene sequencing. 16S rRNA gene sequencing is a powerful technique that offers high taxonomic resolution and data that is reproducible and easily analyzed (Hillis et al. 1996). In addition, large databases (i.e. GenBank) exist against which researchers can compare their findings. Sequencing, however, is expensive, time-intensive, and produces data biased by PCR conditions (Hillis et al. 1996, Suzuki et al. 1996, von Wintzingerode et al. 1997). Examples of termite species that have had their gut microbiota described using 16S rRNA gene sequencing include *Reticulitermes speratus* (Hongoh et al. 2003), *Microcerotermes* spp.

(Hongoh et al. 2005a), *Macrotermes gilvus* (Hongoh et al. 2005b), *Hodotermopsis sjoestedti* (Iida et al. 2000), *Zootermopsis angusticollis* (Wenzel et al. 2002), *Cryptotermes domesticus* (Ohkuma and Kudo 1998), *Nasutitermes lujae* (Paster et al. 1996), *Cubitermes* spp. (Schmitt-Wagner et al. 2003), *C. formosanus* (Shinzato et al. 2005), and *R. santonensis* (Yang et al. 2005).

DNA fingerprinting techniques offer a less-expensive, high-throughput method for studying bacterial assemblages. Fingerprinting techniques, however, offer less taxonomic resolution and few databases exist for data comparisons. Examples of fingerprinting techniques used by termite researchers include terminal restriction fragment length polymorphism analysis (T-RFLP) (Schmitt-Wagner et al. 2003, Hongoh et al. 2005a, 2005b; Nakajima et al. 2005, Noda et al. 2005, Yang et al. 2005), enterobacterial repetitive intergenic consensus-PCR analysis (ERIC-PCR) (Bauer et al. 2000), denaturing gradient gel electrophoresis analysis (DGGE) (Bauer et al. 2000), and amplified rDNA restriction analysis (ARDRA) (Lilburn et al. 1999, Wenzel et al. 2002). Fingerprinting techniques have mainly been used in the study of the termite gut microbiota as clone screening tools. Those clones demonstrating different fingerprints are further analyzed by sequencing. The one exception is T-RFLP analysis which has also been used to identify differences between distinct populations of gut microorganisms (Schmitt-Wagner et al. 2003, Hongoh et al. 2005a, 2005b). One of the drawbacks, however, to T-RFLP analysis is its lack of taxonomic resolution. Since T-RFLP is a community fingerprinting technique that does not target individual sequences and since the original sequences are digested, the exact gene sequence of the individual fingerprinted species remains unknown.

ARDRA is a fingerprinting technique that specifically targets the rRNA gene. Cloned polymerase chain reaction (PCR) products are digested by restriction enzymes and the resultant fragments are visualized via gel electrophoresis. Different fragment patterns (polymorphisms) are interpreted as representing different ribotypes (species). Individual fingerprinting patterns can later be sequenced, if desired, using the original pre-digestion DNA. In addition, ARDRA does not require any specialized equipment.

In this study, I analyzed the usefulness of ARDRA in characterizing the diversity of the gut microbiota in termites using *R. flavipes* as the model termite. Though ARDRA has been utilized before as a pre-sequencing screening tool in termites (Lilburn et al. 1999, Wenzel et al. 2002), I focused on determining its effectiveness as a stand alone tool for characterizing termite gut bacterial diversity and intercolony differences in gut bacterial community structure.

Materials and Methods

Termite collection and identification. *R. flavipes* were collected from three colonies [Pandapas Pond, Boley Fields, and Pilot (respectively termed PP, BF, and PT)] in fallen dead wood in Montgomery County, Virginia, United States. Each colony was located >3 km from all other colonies. In order to avoid the possible effects of laboratory storage on the termite gut microbiota, I used only freshly collected termites for all analyses. Termites from the soldier caste were used to identify the colony as *R. flavipes* (Scheffrahn and Su 1994).

DNA extraction and PCR amplification. I extracted separately the gut bacterial DNA of four samples, two from colony PP (PP-1 and PP-2) and one each from colonies

BF and PT (BF-1 and PT-1). For each DNA extraction, 30 termite workers were washed and their guts were excised using sterilized forceps. I placed the 30 guts in a single 1.5 ml microcentrifuge tube filled with 200 μl of PBS buffer solution [130 mM NaCl, 10 mM sodium phosphate buffer (pH 7.2)] and gently crushed the guts using a sterilized pestle. Gut cells were harvested by centrifugation (5000×*g* for 10 min), decanting the supernatant, and resuspending the pellet in 180 μl of enzymatic lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100). Lysozyme (20 mg ml^{-1}; Fisher Scientific) was mixed into the sample of gut cells and incubated for 30 min at 37°C. After incubation, I added 25 μl of proteinase K and 200 μl of buffer AL (Qiagen) to the gut sample. The mixture was vortexed and incubated for 30 min at 70°C. DNA was then extracted using the Qiagen DNeasy Tissue Kit (Qiagen), starting at step 4 of the "Purification of Total DNA from Animal Tissue" protocol that accompanies the extraction kit.

The 16S rRNA gene was amplified by PCR from the extracted DNA using *Taq* DNA polymerase (Promega) and a GeneAmp 9700 PCR System. Each 40 μl PCR reaction contained 0.8 μl of the DNA extract, 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton® X-100), 2 mM $MgCl_2$, 200 μM of each dNTP, 1.6 U *Taq*, 14.08 μl of ddH_20, and 2 μM of each primer. I used the bacteria-specific PCR primers 41F (Weidner et al. 1996) and 1389R (Marchesi et al. 1998, Osborn et al. 2000). The PCR cycling regime was as follows: 2 min of the initial denaturation at 95°C followed by 24 cycles at 95°C for 30 s, 50°C for 1 min, and 74°C for 4 min, and a final elongation step at 74°C for 10 min. The correct PCR product size (~1.4 kb) was confirmed by electrophoresis on a 1.0 % low-melting-point agarose gel (Amresco). I completed PCR

clean-up with a QIAquick PCR Purification Kit (Qiagen) following manufacturer protocols.

Preparation of clone libraries. Using the TOPO TA Cloning Kit (Invitrogen), PCR products were cloned into pCR®II-TOPO® cloning vectors, and DH5α-T1 strain *Escherichia coli* cells were transformed with the vectors following manufacturer protocols. Clone libraries were then established on selective LB agar plates. I used blue-white screening to select for cells containing plasmids with the intended insert.

PCR amplification of clone inserts. Eighty-one 16S rRNA gene plasmid inserts were amplified by PCR from each sample using *Taq* DNA polymerase (Promega) and a GeneAmp 9700 PCR System. Using a sterilized toothpick, a small amount of each selected clone was placed into a 0.2 ml PCR tube filled with 50 μl of PCR reaction cocktail [1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton® X-100), 2 mM $MgCl_2$, 200 μM of each dNTP, 36.3 μl of ddH_20, and 0.5 μM of each primer (M13F and M13R)]. In order to burst the bacterial cell walls and release the plasmids, the resultant mixture was briefly mixed and incubated in a thermocycler for 10 min at 94ºC. After incubation, I quickly added 1.0 U *Taq* to the reaction cocktail and began the PCR cycling regime. The PCR cycling regime was as follows: 25 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 4 min, and a final elongation step at 72°C for 10 min.

Restriction enzyme digestion and fragment analysis. The PCR products were then individually digested with the four-base cutting restriction enzyme *HhaI* (5'…GCGˇC…3') (Promega). Digestions were performed in 1.5 ml reaction tubes. Each 20 μl reaction contained 7.3 μl sterile water, 2 μl RE 10× buffer C (Promega), 0.2 μl acetylated BSA (Promega), 10 μl DNA, and 0.5 μl restriction enzyme. The resultant

digestion mixture was incubated at 37°C for 2 hr. After incubation 4 µl of 6× loading dye was added (Promega), and the sample was stored in a -20°C freezer until analysis.

I visualized restriction digest fragments on 2.5% NuSieve® GTG® Agarose gels (Cambrex) in 1× TBE buffer. Each gel was run for 1.5 hr at 100V. I then analyzed each gel on a Bio-Rad Molecular Imager Gel Doc XR System and with Quantity One 1-D analysis software (Bio-Rad). Bands sizes were quantified for each lane by comparing them to a standard 50 bp DNA Ladder (BioLabs).

Data Analysis. The purpose of the analysis was two-fold: (1) to determine if ARDRA could be used to distinguish the gut microbiota from different termite colonies and (2) to determine if ARDRA patterns could reliably predict the phylogenetic composition of the gut bacteria. The diversity of my clone libraries was determined by rarefaction analysis (Heck et al. 1975), using the Analytic Rarefaction 1.3 software program (Holland 2006). Rarefaction analysis attempts to estimate the number of species that would have been found had a smaller number of individuals been sampled. The Chao1 nonparametric richness estimator (Chao 1987) was also calculated using the software package EstimateS (Colwell 2005). The Chao1 estimator provided a minimum estimate of the bacterial diversity in the termite gut. Diversity estimation was also conducted using the estimator provided by Curtis et al. (2002) using the program Diversity Calculator v. 53, which is distributed online by the authors (http://people.civil.gla.ac.uk/~sloan/). In this method, the diversity of the termite gut bacteria is estimated using the values N_T, N_{max}, and N_{min}, where N_T is the total number of individuals in the community, N_{max} is the number of individuals in the most abundant species, and N_{min} is the number of individuals in the least abundant species. I assumed

N_{min} was 1. For N_T, I used the population estimations provided by Schultz and Breznak (1978) for the gut bacteria of *R. flavipes*. Schultz and Breznak (1978) estimated the total count of bacteria to be 10^{10} cells ml^{-1} gut fluid or 3×10^6 cells gut^{-1}. This method provides the maximum possible diversity of microorganisms in a community (Curtis et al. 2002). In addition to estimation methods, I also analyzed the data using two diversity indices, including Shannon's diversity index (Shannon and Weaver 1949) and Simpson's index (Simpson 1949).

In order to determine if ARDRA could distinguish differences in the gut microbiota between colonies, I analyzed the data using several different algorithms. I first performed a simple analysis to determine if the proportion of ARDRA profiles shared between two gut samples from the same colony (PP-1 vs. PP-2) was different from the proportion of profiles shared between non-related colonies (PP-1 vs. BF-1, PP-1 vs. PT-1, PP-2 vs. BF-1, and PP-2 vs. PT-1). I used a two-sample test for binomial proportions to test for the differences in the proportions of ARDRA profiles shared between the related colony and non-related colony. SAS 9.1 (SAS Institute 2003) was used in all statistical calculations.

I then analyzed the ARDRA data using several indices of community similarity. I calculated the Bray-Curtis sample dissimilarity index (Magurran 1988, 2004) and the classic Jaccard sample similarity index (Chao et al. 2005). Both of these indices measured the level of similarity in the gut microbiota between two different samples. For the Bray-Curtis index, the values range from 0 to 1, with 0 equating to perfect similarity and 1 equating to no similarity. For the Jaccard index, the values also range from 0 to 1, but 0 is equated with no similarity and 1 with perfect similarity. These similarity indices

were calculated using the software program EstimateS (Colwell 2005). A dendogram was constructed using the Bray-Curtis index of community dissimilarity, using the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) and the software packages PHYLIP (Felsenstein 2005) and TreeView (Page 2001).

I also analyzed the data using a modified version of the technique described by Heyndrickx et al. (1996). A similarity matrix was calculated by comparing the combined ARDRA profile of each gut bacterial population with one another. For each sample comparison, the Dice similarity coefficient (S_D) (Nei and Li 1979) was calculated, according to the formula,

$$S_D = 2\frac{n_{AB}}{(n_A + n_B)}$$

with n_{AB} representing the number of ARDRA profiles common between population A and population B, n_A represents the number of ARDRA profiles in population A, and n_B represents the number of profiles in population B. The values range from 0 to 1, with 0 equating to no similarity and 1 equating to perfect similarity. A dendogram was constructed from the similarity matrix using the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) and the software packages PHYLIP (Felsenstein 2005) and TreeView (Page 2001).

I also wanted to determine if ARDRA patterns could reliably predict the phylogenetic composition of the gut bacteria. The ARDRA patterns from this study were compared to digitally digested termite gut bacteria 16S rRNA gene sequences gleaned from public sequence databases (i.e. GenBank). I digested the 16S rRNA gene sequences using the TACG Restriction Mapping Tool found at

http://biotools.umassmed.edu/tacg/WWWtacg.php. Digitally and experimentally derived ARDRA profiles were then compared by eye and the resultant matches were recorded.

Results and Discussion

Diversity of profiles. I was able to distinguish between different clones using their ARDRA profiles (Fig. 2.1). The diversity of the ARDRA profiles was high. A total of 326 profiles were analyzed from which 175 different ARDRA profiles were found. PP-1, PP-2, PT-1, and BF-1 each had 53, 57, 63, and 64 different profiles, respectively (Table 2.2). The most common profile was profile *ID*, with 23 occurrences (7.0 %) between the four samples (8 in PP-1, 3 in PP-2, and 6 in both PT-1 and BF-1). Of the total profiles found, 122 were found only once (37.4%). This finding suggests that the

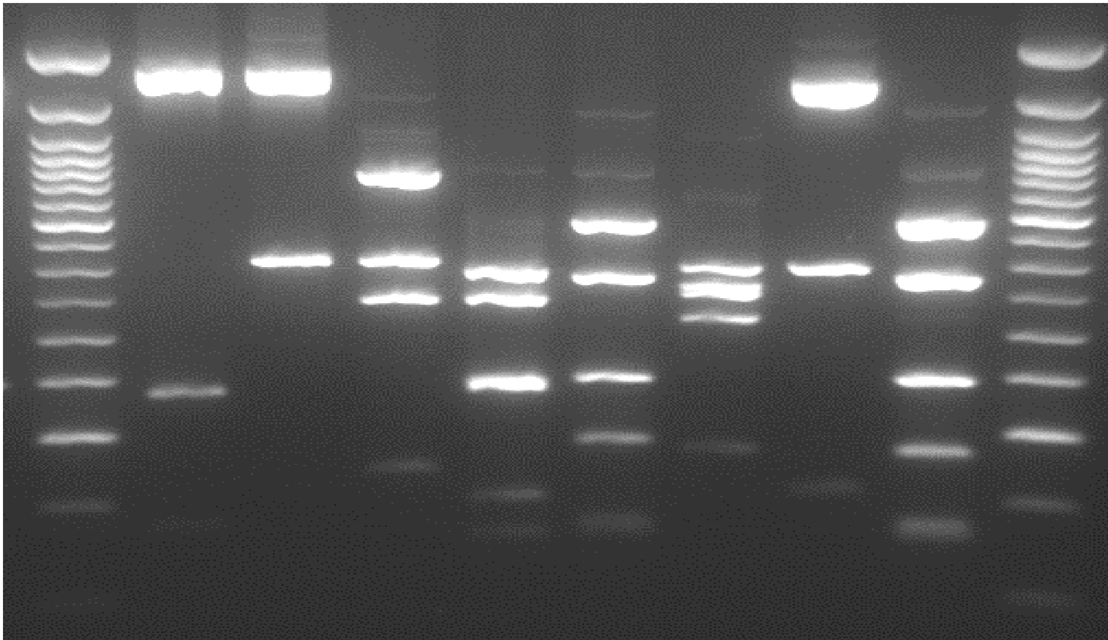

Fig. 2.1. An example of ARDRA profiles. Each lane of the gel is a different digested clone and each band is a DNA fragment. Fragment sizes are determined using the standards found in lanes 1 and 10.

termite gut microbiota is very diverse and includes a preponderance of unique species. Rarefaction analysis (Fig. 2.2) supported the supposition that the termite gut is a very diverse micro-ecosystem that includes many unique species. In rarefaction analysis, if most of the species have been found, the curves would plateau. The rarefaction curves, however, for each colony in this analysis continued their steep climb to the end of the graph indicating that a large proportion of the bacterial species in the termite gut were not sampled.

The supposition that a large majority of the gut bacterial samples were not sampled was supported by the Chao1 and Curtis estimators (Table 2.1). If I used the

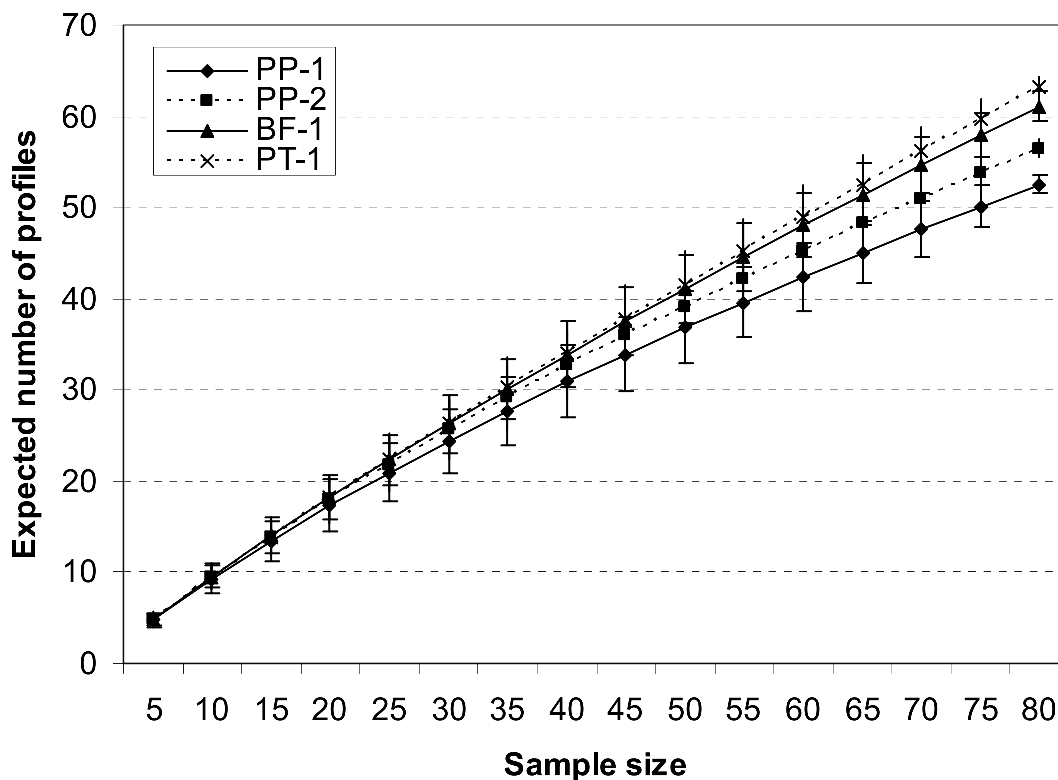

Fig. 2.2. Rarefaction curves for ARDRA profiles of bacteria from PP-1, PP-2, BF-1, and PT-1. The error bars represent the 95% confidence level of the expected number of profiles for each sample size.

Table 2.1. Estimations of the diversity of the bacteria in the termite gut using the Curtis and Chao1 estimators.

Sample	Estimator statistics				
	N	Curtis (species gut^{-1})	Chao1 mean	Chao1 95% CI lower bound	Chao1 95% CI upper bound
PP-1	81	778	139.87	97.14	223.04
PP-2	81	1335	154.00	97.14	289.39
PT-1	83	1122	287.33	151.56	628.88
BF-1	81	1092	1687.50	368.12	8712.05

Chao1 estimator means to predict a minimum number of species, there are at least 140 species. The Curtis estimator, on the other hand, predicted that between 778 to 1,335 species of bacteria are present in each termite gut. This result corresponds closely to the figures reported by Hongoh et al. (2003). Hongoh et al. (2003) found a total of 1,056 phylotypes (similar to species) in *R. speratus* and estimated that 700 phylotypes are present per termite gut. The higher species richness estimations in *R. flavipes* are either because (1) the gut of *R. flavipes* is more diverse or (2) ARDRA data, due to their lack of phylogenetic resolution compared to sequence data, are not as accurate.

The indices of diversity I used provided useful information about the levels of diversity within the different samples. Shannon's diversity index measures both species richness and evenness. Higher values are interpreted as higher diversity (with ~4.5 as the upper boundary and 0.0 as the lower boundary) (Shannon and Weaver 1949). The two inter-colony samples, PP-1 and PP-2, both resulted in similar, lower measures of

Table 2.2. Indices of diversity for each of the samples PP-1, PP-2, PT-1, and BF-1.

Measurement	Sample			
	PP-1	PP-2	PT-1	BF-1
# species (S)	53	57	63	64
Shannon's diversity index	3.77	3.89	4.00	4.00
Simpson's index	0.98	0.99	0.99	0.99

Table 2.3. Results of the two-sample test for binomial proportions.

Intracolony comparison	Intercolony comparisons	Proportion shared		Statistics	
		Intracolony	Intercolony	*Z*	*P*
PP-1 vs. PP-2	PP-1 vs. PT-1	14.8	11.5	0.82	<0.41
	PP-1 vs. BF-1		10.4	1.08	<0.28
	PP-2 vs. PT-1		14.2	0.24	<0.81
	PP-2 vs. BF-1		16.3	-0.17	<0.86

diversity, while the other samples, PT-1 and BF-1, resulted in the same, higher index (4.0) (Table 2.2).

The Simpson index estimates the probability of picking two organisms at random that are of different species. This index tends to be weighted toward the abundance of the most common species and as such is a type of dominance measure. As the value for the Simpson index increases, the probability of any two samples being of the same species decreases (Simpson 1949). All Simpson index values were high (≥0.98) (Table 2.2). Thus the probability of choosing two bacteria from the termite gut and they being of the same species is ≤2%. This result is indicative of the high species diversity in the termite gut for each sample.

The utility of ARDRA to differentiate between the gut microbiotas from different termite colonies was limited. The intracolony comparison (PP-1 vs. PP-2) resulted in a

Table 2.4. Similarity/dissimilarity indices of termite gut bacterial ARDRA profiles for inter- and intracolony comparisons.

Comparison	Similarity/dissimilarity index		
	Bray-Curtis	Jaccard	Dice similarity coefficient
PP-1 vs. PP-2	0.247	0.146	0.255
PP-1 vs. PT-1	0.268	0.115	0.207
PP-1 vs. BF-1	0.261	0.105	0.188
PP-2 vs. PT-1	0.268	0.143	0.250
PP-2 vs. BF-1	0.286	0.165	0.281

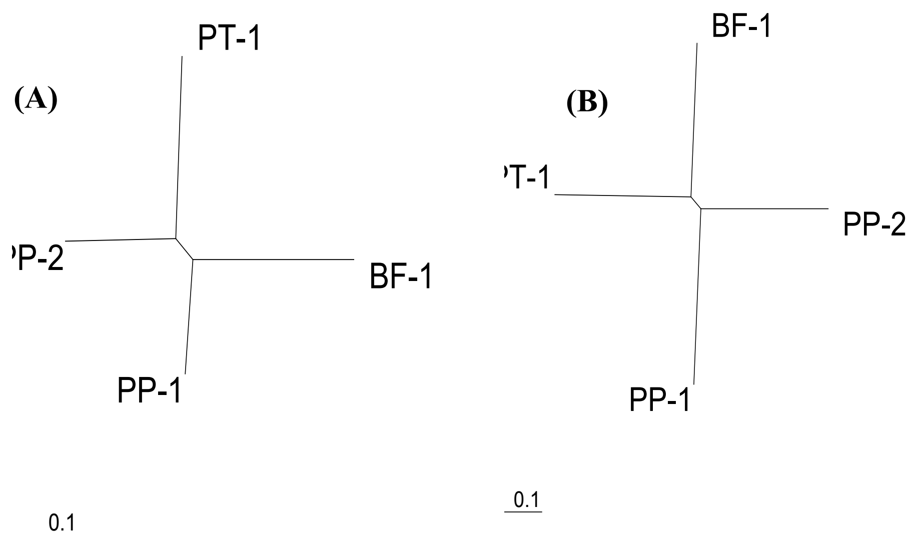

Fig. 2.3. Unrooted dendograms constructed from the Bray-Curtis sample dissimilarity data (A) and the Dice similarity coefficient data (B). Dendograms were constructed using the neighbor-joining algorithm. The scale bar represents the percentage of dissimilarity (0.1=10%).

sharing of 14.8% of the ARDRA profiles. All intercolony comparisons, with the exception of PP-2 vs. BF-1, shared smaller proportions of their ARDRA profiles. The two-sample test for binomial proportions, however, found no significant differences in the proportions of shared ARDRA profiles between intra- and intercolony comparisons (Table 2.3).

The similarity indices were also inconclusive (Table 2.4). The Bray-Curtis sample dissimilarity index suggested that the intracolony comparison (PP-1 vs. PP-2) was more similar than any of the intercolony comparisons. PP-1 vs. PP-2 had a lower index (0.247) than any of the intercolony comparisons. These data support the conclusion that the Bray-Curtis sample dissimilarity index could be used to distinguish termite colonies by their gut bacteria ARDRA profiles. The neighbor-joining dendogram (Fig. 2.3) lent some support to this conclusion since PP-1 and PP-2 were closer to one another than to the other samples.

The remaining similarity indices, however, gave mixed results (Table 2.4). Both the Jaccard index and the Dice similarity coefficient resulted in similar data patterns. Both indices did show the intracolony comparison (PP-1 vs. PP-2) as having more similarity than three of the intercolony comparisons (PP-1 vs. PT-1, PP-1 vs. BF-1, and PP-2 vs. PT-1) but less than one of the intercolony comparisons (PP-2 vs. BF-1). The dendogram constructed using the Dice similarity coefficients illustrated the discrepancy (Fig. 2.3). Little difference is observed in the distance between intra- and intercolony samples.

The inconsistency of these results may be due to one of three possibilities. Either (1) the sample sizes (81-83 clones) were too small to accurately and consistently detect differences between the samples, (2) there are no significant differences in the gut bacterial composition between colonies of *R. flavipes*, or (3) ARDRA data are inherently not useful for detecting differences in very diverse populations. The first possibility regarding sample size is a very real possibility. In other words, more ARDRA profiles need to be performed for each colony. Since the Simpson's index for all samples was ≥98 (Table 2.2) and the rarefaction curves were steep (Fig. 2.2) it is very apparent that the termite gut bacteria are extremely diverse. It would require a very large sample size in order to capture a good representative sample of the true diversity. Determining sample size is a very important issue because analysis of samples that are too large is costly with respect to time, resources, and money, while samples that are too small may lead to inaccurate results (Ott 1992). Further studies measuring the effects of sample size on the effectiveness of the indices I used are necessary.

The second possibility regarding no significant differences in the gut bacterial composition between colonies of *R. flavipes* is highly unlikely. Several authors have demonstrated that the gut microbiota of other termite species varies dramatically both between and within colonies. Hongoh et al. (2005a) showed that the gut microbiota in *R. speratus* is similar yet significantly different between termite colonies. Hongoh at el. (2005b) also demonstrated that the gut microbiota varies between different castes and different ages in the termite *Macrotermes gilvus*. Thus it is unlikely that the gut bacterial species composition between different colonies of *R. flavipes* is the same.

Regarding the possibility that ARDRA is not useful, several other fingerprinting techniques have been used “successfully” in termites to detect differences in the bacterial species complex in the termite gut. These techniques include the use of terminal restriction fragment length polymorphism analysis (T-RFLP) (e.g. Hongoh et al. 2005b) and denaturing gradient gel electrophoresis analysis (DGGE) (e.g. Bauer et al. 2000). T-RFLP analysis measures the genetic diversity of entire populations at once, but only uses a terminal fragment of the targeted gene. If two species have similar terminal fragment lengths, they can be mistaken for the same species. Thus an underestimation of the true diversity can occur with T-RFLP analysis. In addition, there is not an easy way to retrieve the entire sequence of the fragments being analyzed. DGGE is a more sensitive fingerprinting technique that utilizes multiple fragments. Using DGGE, infrequently occurring bacterial species can be more easily detected (Muyzer and Smalla 1998). In addition, the entire sequence of the sample in question can be retrieved from the gel and sequenced. DGGE, however, requires additional specialized equipment. ARDRA does not require additional specialized equipment, it offers higher taxonomic resolution, it

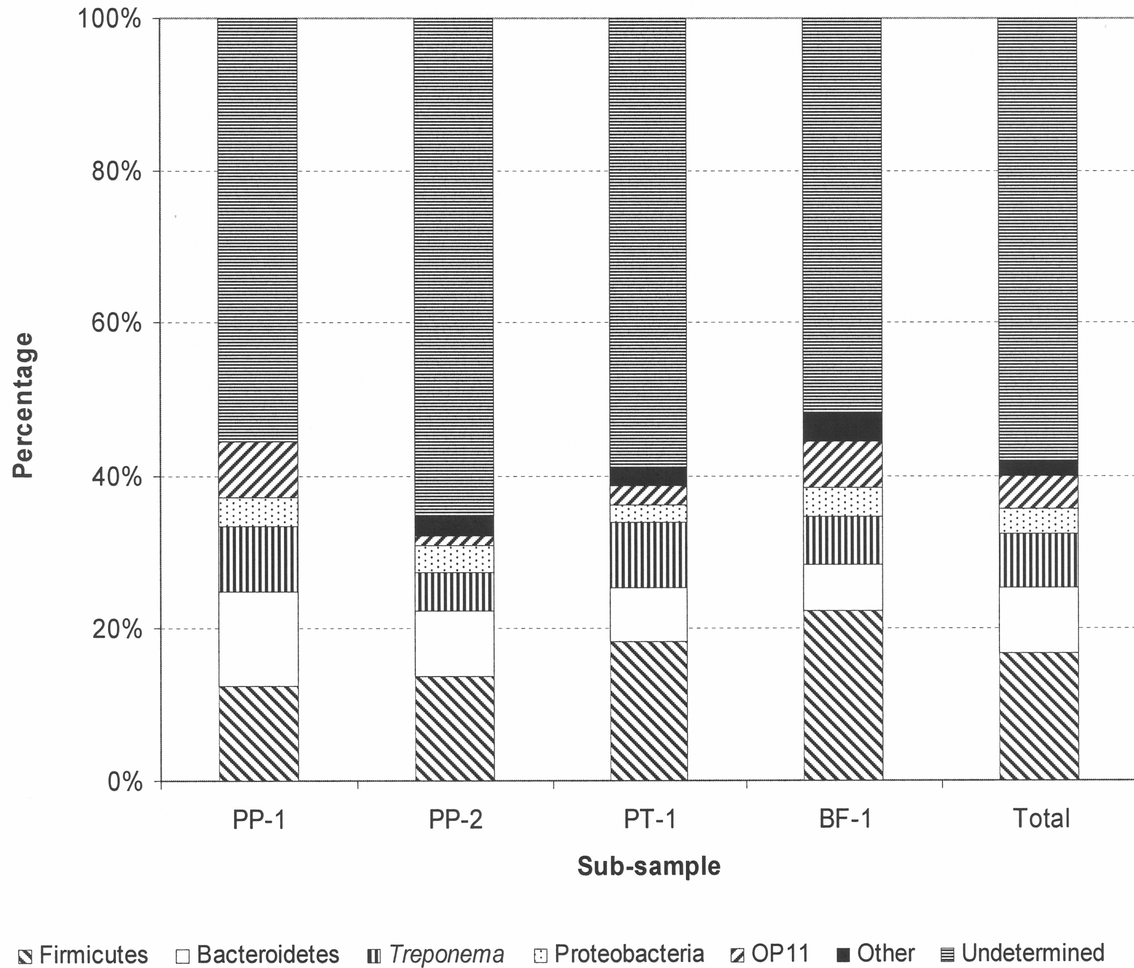

Fig. 2.4. Taxonomic composition of termite gut bacterial ARDRA profiles taken from *R. flavipes*. The taxonomic assignments were accomplished using digitally digested 16S rRNA sequences from *R. speratus* (see Table 2.4).

allows for the retrieval of the sample sequence, and it gives consistent results. Thus ARDRA is an excellent candidate technique for studying the termite gut microbiota.

To demonstrate the effectiveness of ARDRA for describing the gut bacteria taxonomic composition, I compared the experimentally derived ARDRA profiles with the digitally determined profiles of termite gut bacterial sequences from public databases. The results are summarized in Fig. 2.4 and Table 2.5. The ARDRA profiles of 326 clones were analyzed. I was able to match 137 of the experimentally derived profiles

with digitally derived profiles. The remaining 189 profiles did not match with any previously described sequences. The fact that most of the profiles (58.0%) did not match with any of the previously described sequences supports the idea that much of the diversity of the termite gut remains undescribed. The fact that most of the ARDRA profiles (136 total) did not match with the digital ARDRA profiles of sequences from other termite species (Table 2.5) also supports the idea that different species of termites harbor different species of symbiotic bacteria (Hongoh et al. 2005a). Most of the digitally determined profiles originated from *R. speratus*.

A comparison of the proportions of the different taxonomic units from this study (Fig. 2.4) with those described in Hongoh et al. (2003) shows major differences. Apart from the undetermined profiles, the experimentally derived ARDRA profiles that matched the digital profiles for bacteria in the phylum Firmicutes accounted for the greatest proportion of profiles (16.6%). Hongoh et al. (2003) concluded that the *Firmicutes* (represented by Clostridia in their study) were usually the third largest group of bacteria found in the termite gut, behind the *Bacteroidetes* and the *Spirochaetes*. My study also showed that only 7.1% of the ARDRA profiles matched with *Spirochaetes* (*Treponema*) sequences from Hongoh et al. (2003). This percentage of *Spirochaetes* is a major difference from Hongoh et al. (2003). Hongoh et al. (2003) determined that *Spirochaetes* made up half of the total clones described. It was also surprising to find a general lack of ARDRA profiles matching known "*Endomicrobia*" sequences. "*Endomicrobia*" made up a large percentage of the clones in *R. speratus* (Hongoh et al. 2003) but I only found two ARDRA profiles that matched previous "*Endomicrobia*" sequences.

These comparisons, however, between my ARDRA data and the digital ARDRA profiles of sequences from public sequence databases must be made with great caution. It is important to note that a majority of my experimentally derived ARDRA profiles were undetermined (Fig. 2.4). Determining the taxonomic status of these undetermined ARDRA profiles may have completely changed the proportional representation of the taxonomic units. For example, the lack of experimentally derived ARDRA profiles assigned to *Treponema* and "*Endomicrobia*" may have been due to a lack of representative sequences in the public databases from *R. flavipes*. If the termite gut bacteria are different between different species of termites, as suggested by Hongoh et al. (2005a), then the lack of representative sequences from *R. flavipes* would have resulted in a lack of digital ARDRA profiles against which to compare my experimentally derived ARDRA profiles. Thus the experimental ARDRA profiles that should have represented *Treponema* and "*Endomicrobia*" would have been labeled "undetermined" in the current study. If future studies on the gut microbiota of termites utilize ARDRA, it is recommended that an extensive 16S rRNA gene sequence database first be established for the termite species of interest. Such a database would then provide researchers with the appropriate digital data necessary for making taxonomic comparisons.

Table 2.5. Assignment of clones from this study to known 16S rRNA bacterial sequences based on ARDRA profiles. The table matches the ARDRA profiles of clones from this study to digitally generated ARDRA profiles of known 16S rRNA gene sequences. All sequences with accession #'s beginning with "AB" were from *R. speratus*. Those beginning with "DQ" were from *R. flavipes*.

Profile name	Representative 16S rRNA gene sequence (accession # | classification)	Clones assigned to profile	ARDRA Profile (Band Lengths)
AA	AB089105|delta proteobacterium	DA6	277, 271, 255, 207, 166, 165, 137, 91, 19, 2
AE	AB089047|Clostridiaceae bacterium	BB16, BC17	348, 256, 214, 184, 174, 165, 148, 70, 22
AM	AB088985|Clostridiales bacterium	BA47, BB15	390, 347, 258, 225, 151, 128, 56
AN	AB089019|Clostridiales bacterium	CD5	390, 347, 258, 184, 151, 112, 107, 2
AP	AB089057|*Mycoplasma* sp.	BB12	394, 361, 280, 270, 250
AW	AB089106|delta proteobacterium	AD11, DC2	408, 397, 216, 206, 169, 135, 62
BA	AB089098|alpha proteobacterium	AA38, CD32	410, 341, 259, 210, 184, 135
BB	AB089012|Clostridiales bacterium	AD5	410, 345, 334, 301, 184
BT	AB088934|Bacteroidaceae bacterium	BA35, BA46, DD23	454, 377, 247, 171, 149, 135, 31, 14
BV	AB089072|Actinobacteridae bacterium	CB20	456, 411, 254, 157, 156, 116, 42
CI	AB088917|Bacteroidaceae bacterium	AA25, AA30, AB20, AD24, BC5, CA9, CD6, CD9, DC6	485, 377, 247, 173, 149, 135, 14
CL	AB088931|Bacteroidaceae bacterium	AC1, AC13, AC5, AD15, BA17, BA50, BD1, CD14, DA10	511, 375, 247, 178, 149, 113, 14
CQ	AB088919|Bacteroidaceae bacterium	AC3, AD6	525, 472, 247, 177, 163
CT	AB089108|delta proteobacterium	AB14, BC11	531, 276, 273, 206, 138, 137, 31
CY	DQ009675|gamma proteobacterium	BD12	531, 451, 273, 197, 136
DB	AB088894|*Treponema* sp.	BB6, CA7, DC16	534, 273, 249, 184, 149, 116, 70, 13
DD	AB088857|*Treponema* sp.	AA36, AB1, AD23, BD15, CA10, CC17, DA19, DB2, DC13	534, 343, 249, 184, 150, 113, 13
DN	AB089112|epsilon proteobacterium	BA26	567, 350, 265, 178, 106, 58, 42
DR	AB088882|*Treponema* sp.	AA37	590, 389, 249, 149, 116, 82, 13
DT	AB089097|alpha proteobacterium	CB8	590, 411, 205, 184, 139

Table 2.5. Assignment of clones from this study to known 16S rRNA bacterial sequences based on ARDRA profiles. The table matches the ARDRA profiles of clones from this study to digitally generated ARDRA profiles of known 16S rRNA gene sequences. All sequences with accession #'s beginning with "AB" were from *R. speratus*. Those beginning with "DQ" were from *R. flavipes*.

Profile name	Representative 16S rRNA gene sequence (accession # | classification)	Clones assigned to profile	ARDRA Profile (Band Lengths)
DU	AB088867.1|*Treponema* sp.	BD6	590, 471, 249, 149, 116, 13
EA	AB088943|Bacteroidaceae bacterium	CA22, DD14	620, 375, 247, 166, 149, 14
EC	AB089016|Clostridiales bacterium	CD4	621, 409, 345, 184
EE	AB089066|Unknown bacterium	DA15	623, 507, 411, 27
EL	AB089100|beta proteobacterium	DA18	651, 474, 409, 54
EN	AB089029|Clostridiaceae bacterium	CB20	654, 403, 348, 184
ES	AB089063|*Lactococcus* sp.	DB3	668, 527, 408
EU	AB089036|Clostridiaceae bacterium	DC1	673, 475, 250, 153, 54
FE	AB089073|Actinobacteridae bacterium	DB5	706, 449, 263, 146
FG	AB088955|Eubacteriaceae bacterium	DD25	711, 441, 409
FH	AB089040|Clostridiaceae bacterium	DD8	711, 477, 405
GB	AB088878|*Treponema* sp.	CA23	842, 248, 149, 141, 116, 70, 13, 8
GC	AB088953|Clostridiaceae bacterium	AC17	845, 403, 292
GK	AB088907|*Treponema* sp.	BA37, CB19, CB3	889, 249, 164, 149, 116, 13, 8
GP	AB088972|Eubacteriaceae bacterium	CA14, DC11	901, 410, 270
GU	AB088913|*Treponema* sp.	DA5	991, 249, 149, 116, 70, 13
GW	AB088929|Bacteroidaceae bacterium	BD17, CD20	988, 241, 185, 169
HA	AB088942|Bacteroidaceae bacterium	DC4	998, 175, 149, 137, 110, 14
HB	AB089052|Termite group 1 bacterium	CD11	1021, 408, 138
HC	AB088983|Clostridiaceae bacterium	BB5, BD2, CB13, CC2, DA11, DC3, DC8	1035, 407, 136, 2
HD	AB088887|*Treponema* sp.	AA31	1061, 177, 149, 116, 72, 13
HM	AB088876|*Treponema* sp.	AC12, AD19, CD1	1091, 249, 149, 70, 13
HN	AB089114|OP11 bacterium	AB16, AB17, AC15	1118, 394
HO	AB088951|Clostridiaceae bacterium	BC6, CA15, DD17	1136, 407

Table 2.5. Assignment of clones from this study to known 16S rRNA bacterial sequences based on ARDRA profiles. The table matches the ARDRA profiles of clones from this study to digitally generated ARDRA profiles of known 16S rRNA gene sequences. All sequences with accession #'s beginning with "AB" were from *R. speratus*. Those beginning with "DQ" were from *R. flavipes*.

Profile name	Representative 16S rRNA gene sequence (accession # | classification)	Clones assigned to profile	ARDRA Profile (Band Lengths)
HP	AB089125|OP11 bacterium	AC2, AC20, AC4, BC2, CA8, CB16, DA12, DA14, DA4, DC7, DD2	1141, 394
HQ	AB089027|Clostridiaceae bacterium	CA16, DD11	1157, 411
HS	DQ009685|Clostridiales bacterium	BC7	1147, 272, 136
HW	AB089048|Termite group 1 bacterium	BC3	1160, 408
HX	AB089064|Unknown bacterium	DC15	1166, 410
IA	AB088950|Eubacteriaceae bacterium	CD2	1169, 223, 137, 50
IB	AB088968|Clostridiaceae bacterium	DA8, DD7	1170, 402
ID	AB088966|Clostridiaceae bacterium	AA28, AA29, AB10, AB13, AB19, AB4, AC16, AD20, BC14, BC9, BD21, CA12, CA19, CB6, CC14, CD27, CD3, DB1, DB13, DC12, DD22, DD28, DD29	1191, 408
MA to TG	None	189 clones total	Varied

References Cited

Bauer, S., A. Tholen, J. Overmann, and A. Brune. 2000. Characterization of abundance and diversity of lactic acid bacteria in the hindgut of wood- and soil-feeding termites by molecular and culture-dependent techniques. Arch. Microbiol. 173: 126-137.

Benemann, J. R. 1973. Nitrogen fixation in termites. Science 181: 164-165.

Breznak, J.A., W. J. Brill, J. W. Mertins, and H. C. Coppel. 1973. Nitrogen fixation in termites. Nature 244: 577-580.

Breznak, J. A., and J. M. Switzer. 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl. Environ. Microbiol. 52: 623–630.

Chao, A. 1987. Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43: 783-791.

Chao, A., R. L. Chazdon, R. K. Colwell, and T.–J. Shen. 2005. A new statistical approach to assessing compositional similarity based on incidence and abundance data. Ecol. Lett. 8: 148-159.

Colwell, R. K. 2005. EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5. User's Guide and application published at: http://purl.oclc.org/estimates.

Curtis, T. P., W. T. Sloan, and J. W. Scannell. 2002. Estimating prokaryote diversity and its limits. Proc. Natl. Acad. Sci. USA 99: 10494-10499.

Edwards, R., and A. E. Mill. 1986. Termites in buildings: their biology and control. Rentokill Limited, W. Sussex, UK.

Felsenstein, J. 2005. PHYLIP: phylogeny inference package. Version 3.65. University of Washington, Seattle, WA.

French, J. R. J., G. L. Turner, and J. F. Bradbury. 1976. Nitrogen fixation by bacteria from the hindgut of termites. J. Gen. Microbiol. 95: 202-206.

Heck, K.L., Jr., G. Van Belle, and D. Simberloff. 1975. Explicit calculation of the rarefaction diversity measurement and the determination of sufficient sample size. Ecology 56:1459-1461.

Heyndrickx, M., L. Vauterin, P. Vandamme, K. Kersters, and P. De Vos. 1996. Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. J. Microbiol. Meth. 26: 247-259.

Hillis, D. M., C. Moritz, and B. K. Mable. 1996. Molecular systematics, 2nd edition. Sinauer Associates, Sunderland, MA.

Holland, S. M. 2006. Analytic rarefaction user's manual, version 1.3. Steven M. Holland, University of Georgia, GA.

Hongoh, Y., M. Ohkuma, and T. Kudo. 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol. Ecol. 44:231-242.

Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, and T. Kudo. 2005a. Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl. Environ. Microbiol. 71: 6590-6599.

Hongoh, Y., L. Ekpornprasit, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, N. Noparatnaraporn, and T. Kudo. 2005b. Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. Mol. Ecol. 15: 505-516.

Honigberg, B. M. 1969. Protozoa associated with termites and their role in digestion, pp. 1-36. *In* K. Krishna and F. M. Weesner (eds.), Biology of termites, vol. 2, Academic Press, New York, NY.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180: 4765-4774.

Iida, T., M. Ohkuma, K. Ohtoko, and T. Kudo. 2000. Symbiotic spirochetes in the termite hindgut: phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol. Ecol. 34: 17-26.

Kellenberger, E. 2001. Exploring the unknown: the silent revolution of microbiology. EMBO Rep. 2: 5-7.

Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283: 686-689.

Lilburn, T. G., T. M. Schmidt, and J. A. Breznak. 1999. Phylogenetic diversity of termite gut spirochaetes. Environ. Microbiol. 1: 331-345.

Magurran, A. E. 1988. Ecological diversity and its measurement. Princeton University Press, Princeton, NJ.

Magurran, A. E. 2004. Measuring biological diversity. Blackwell Publishing, Boston, MA.

Marchesi, J. R., T. Sato, A, J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64:795-799.

Muyzer, G, and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek 73: 127-141.

Nakajima, H., Y. Hongoh, R. Usami, T. Kudo, and M. Ohkuma. 2005. Spatial distribution of bacterial phylotypes in the gut of the termite *Reticulitermes speratus* and the bacterial community colonizing the gut epithelium. FEMS Microbiol. Ecol. 54: 247-255.

Nei, M., and W.-H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269-5273.

Noda, S., T. Iida, O. Kitade, H. Nakajima, T. Kudo, and Moriya Ohkuma. 2005. Endosymbiotic *Bacteroidales* bacteria of the flagellated protest *Pseutotrichonympha grassii* in the gut of the termite *Coptotermes formosanus*. Appl. Environ. Microbiol. 71: 8811-8817.

Ohkuma, M., and T. Kudo. 1998. Phylogenetic analysis of the intestinal microflora of the termite *Cryptotermes domesticus*. FEMS Microbiol. Letters 164: 389-395.

Osborn, A. M., E. R. Moore, and K. N. Timmis. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2:39-50.

Ott, L. 1992. An introduction to statistical methods and data analysis, 4th ed. Wadsworth Publishing Co., Belmont, CA.

Page, R. D. M. 2001. TreeView 1.6.6. University of Glasgow, Glasgow, UK.

Paster, B. J., F. E. Dewhirst, S. M. Cooke, V. Fussing, L. K. Poulsen, and J. A. Breznak. 1996. Phylogeny of not-yet-cultured spirochetes from termite guts. Appl. Environ. Microbiol. 62: 347-352.

Pinto, L. J. 1981. The structural pest control industry: description and impact on the nation. National Pest Control Association, Vienna, VA.

Potrikus, C. J, and J. A. Breznak. 1980. Uric acid degrading bacteria in guts of termites (*Reticulitermes flavipes* [Kollar]). Appl. Environ. Microbiol. 40: 117-124.

Potrikus, C. J., and J. A. Breznak. 1981. Gut bacteria recycle uric acid nitrogen in termites: a strategy for nutrient conservation. Proc. Nat. Acad. Sci. USA 78: 4601-4605.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

SAS Institute. 2003. SAS 9.1.3 help and documentation. SAS Institute, Cary, NC.

Scheffrahn, R. H., and N.–Y. Su. 1994. Key to soldier and winged adult termites (Isoptera) of Florida. Fla. Entomol. 77: 460-474.

Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune. 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). Appl. Environ. Microbiol. 69: 6018-6024.

Schultz, J. E., and J. A. Breznak. 1978. Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. Appl. Environ. Microbiol. 35: 930-936.

Shannon, C. E., and W. Weaver. 1949. The mathematical theory of communication. University of Illinois Press, Urbana, IL.

Sharma, R., R. Ranjan, R. K. Kapardar, and A. Grover. 2005. 'Unculturable' bacterial diversity: an untapped resource. Curr. Sci. 89: 72-77.

Shinzato, N., M. Muramatsu, T. Matsui, and Y. Watanabe. 2005. Molecular phylogenetic diversity of the bacterial community in the gut of the termite *Coptotermes formosanus*. Biosci. Biotechnol. Biochem. 69: 1145-1155.

Simpson, E. H. 1949. Measurement of diversity. Nature 163: 688.

Su, N.–Y., and R. H. Scheffrahn. 1990. Economically important termites in the United States and their control. Sociobiology 17: 17-94.

Suzuki, M. T., and S. J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62: 625-630.

Von Wintzingerode, F., U. B. Gobel, and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21: 213-229.

Weidner, S., W. Arnold, and A. Puhler. 1996. Diversity of uncultured microorganisms associated with the seagrass *Halophila stipulacea* estimated by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. 62:766-771.

Wenzel, M., I. Schönig, M. Berchtold, P. Kämpfer, and H. König. 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. J. Appl. Microbiol. 92: 32-40.

Yang, H., D. Schmitt-Wagner, U. Stingl, and A. Brune. 2005. Niche heterogeneity determines bacterial community structure in the termite gut (*Reticulitermes santonensis*). Environ. Microbiol. 7: 916-932.

Chapter Three
Diversity of the Gut Microbiota of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) as described by 16S rRNA Gene Sequencing and Amplified rDNA Restriction Analysis (ARDRA)

Introduction

The termite gut is a complex micro-ecosystem consisting of a diverse array of protozoa, bacteria, archaea, and fungi. The relationship between the termite host and its gut symbionts is a form of obligatory mutualism. The microorganisms serve a variety of important functions for the host termite, including cellulase production (Slaytor 1992), acetogenesis (Breznak and Kane 1990, Leadbetter et al. 1999), and nitrogen acquisition (Breznak et al. 1973). In return, the termite gut provides an optimal environment for growth and reproduction, and the microorganisms receive a steady influx of nutrients.

Because termite gut bacteria require strict environmental conditions for growth and reproduction, culturing techniques are often inadequate for studying these organisms (Sharma et al. 2005). The lack of effective culturing techniques, in turn, has hampered phylogenetic identification of the termite gut bacteria. Culture-independent methods, such as molecular approaches, provide an effective alternative for studying the phylogenetic diversity of termite gut bacteria. In particular, the analysis of 16S rRNA gene sequence data and fingerprinting data [i.e. terminal restriction fragment length polymorphism analysis (T-RFLP)] has recently produced a steady flow of information on the species diversity of the gut bacteria in a variety of termite species (To et al. 1980, Ohkuma and Kudo 1996, Paster et al. 1996, Keeling et al. 1998, Kudo et al. 1998, Ohkuma and Kudo 1998, Lilburn et al. 1999, Breznak 2000, Brauman et al. 2001, Hongoh et al. 2003, Schmitt-Wagner et al. 2003, Shinzato et al. 2005, Hongoh et al.

2006). Relatively little work has been done to phylogenetically characterize the bacteria found in the gut of *R. flavipes*. *R. flavipes* studies have focused on sub-groups of bacteria, such as spirochetes (Lilburn et al. 1999), lactic acid bacteria (Bauer et al. 2000), and the newly described phylum "*Endomicrobia"* (Stingl et al. 2005).

Recent research has revealed that many of these uncultivable bacteria, including those found in termites, are undescribed species belonging to new phylogenetic clades within Eubacteria and Archaea (Hugenholtz et al. 1998, Hongoh et al. 2005). Thus the ecological impacts and biotechnological applications of this unculturable majority remain relatively unexplored (Kellenberger 2001). If there are thousands of undescribed termite gut microorganisms and each undescribed species contains thousands of undescribed genes, what other ecological and biotechnological discoveries await those who study the termite-symbiont system? For example, Physics Nobel laureate Steven Chu recently suggested that the termite gut may hold an important key in solving the world's energy problems (Chu 2005). Chu argued that evolution has produced in termites a carbon-neutral method for turning cellulose into ethanol. Some of the bacteria in the termite gut efficiently take low-grade fuel sources, such as plant matter, and convert them into fuel that releases no more carbon than it took to produce it. Another recent example has been the finding that the termite gut microbiota has coevolved with its termite host (Hongoh et al. 2005). Using eight Asian termite species, the authors showed that the gut microbiota was specific for each termite genera they studied. Both Chu (2005) and Hongoh et al. (2005) argued that in order to ascertain the biotechnological and ecological impacts of the termite gut microbiota, more information is need about its diversity.

This study examines 16S rRNA gene sequence and amplified ribosomal DNA restriction analysis (ARDRA) data of symbiotic bacteria from a wide range of phylogenetic groups in the gut of *R. flavipes*. The purpose of this study is to provide a springboard for future analyses of the diversity of the gut microbiota of this economically important termite.

Materials and Methods

Termite collection and identification. For the 16S rRNA gene sequencing study, *R. flavipes* were collected from a single colony in fallen dead wood from Prince George County, Virginia, United States. For the ARDRA study, *R. flavipes* were collected from fallen dead wood at four sites (termed PPA, PPB, BF, and PT) in Montgomery County, Virginia, United States. In order to avoid the possible effects of laboratory storage on the termite gut microbiota, I used only freshly collected termites for all analyses. Termites from the soldier caste were used to positively identify the colony as *R. flavipes* (Scheffrahn and Su 1994).

DNA extraction, PCR, and Cloning. DNA extraction was performed individually for termites from each collection site. For each DNA extraction, 30 termite workers were washed and their guts were excised using sterilized forceps. The 30 guts were then placed in a single 1.5 ml microcentrifuge tube filled with 200 µl of PBS buffer solution [130 mM NaCl, 10 mM sodium phosphate buffer (pH 7.2)] and gently crushed them using a sterilized pestle. Gut cells were harvested by centrifugation (5000×*g* for 10 min), decanting the supernatant, and resuspending the pellet in 180 µl of enzymatic lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100). Lysozyme (20 mg

ml^{-1}; Fisher Scientific) was mixed into the sample of gut cells and incubated for 30 min at 37°C. After incubation, 25 μl of proteinase K and 200 μl of buffer AL (Qiagen) was added to the gut sample and the mixture was vortexed and incubated for 30 min at 70°C. DNA was then extracted using the Qiagen DNeasy Tissue Kit (Qiagen), starting at step 4 of the "Purification of Total DNA from Animal Tissue" protocol that accompanies the extraction kit.

The 16S rRNA gene for each DNA extraction was amplified separately by polymerase chain reaction (PCR) from the extracted DNA using *Taq* DNA polymerase (Promega) and a GeneAmp 9700 PCR System. Each 40 μl PCR reaction contained 0.8 μl of the DNA extract, 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton® X-100), 2 mM $MgCl_2$, 200 μM of each dNTP, 1.6 U *Taq*, 14.08 μl of ddH_20, and 2 μM of each primer. I used the bacteria-specific PCR primers 41F (Weidner et al. 1996) and 1389R (Marchesi et al. 1998, Osborn et al. 2000). The PCR cycling regime was as follows: 2 min of the initial denaturation at 95°C followed by 24 cycles at 95°C for 30 s, 50°C for 1 min, and 74°C for 4 min, and a final elongation step at 74°C for 10 min. The correct PCR product size (~1.4 kb) was confirmed by electrophoresis on a 1.0 % low-melting-point agarose gel (Amresco). I completed PCR clean-up with a QIAquick PCR Purification Kit (Qiagen) following manufacturer protocols.

Using the TOPO TA Cloning Kit (Invitrogen), PCR products for each site were separately cloned into pCR®II-TOPO® cloning vectors, and DH5α-T1 strain *Escherichia coli* cells were transformed with the vectors following manufacturer protocols. Clone libraries representing each site were then established on selective LB agar plates. I used blue-white screening to select for cells containing plasmids with the intended insert.

16S rRNA sequencing experiment. Selected cells were grown overnight in ampicillin selective LB broth. Miniprep cleanup of resulting colonies was accomplished using the QIAprep Spin Miniprep Kit (Qiagen) following manufacturer protocols.

Forty-two clones were sequenced. DNA sequencing was performed at the Virginia Bioinformatics Institute (VBI) Core Laboratory Facility (Blacksburg, Virginia), and at the GeneLab/BioMed facility within the Louisiana State University School of Veterinary Medicine (Baton Rouge, Louisiana). Approximately 1200-1400 bp [corresponding to positions 22-1400 in *E. coli* (J01695)(Brosius et al. 1978)] of the clones were sequenced using the Big-Dye Terminator Cycle Sequencing Kit (Perkin-Elmer), an ABI 370 or 3700 genetic analyzer, and primers T7, 41F, and 1389R. I checked all sequences for chimeric artifacts using the Check-Chimera program on the Ribosomal Database Project (RDP) web site (Cole et al. 2005). A search for similar sequences using BLASTN V2.2 (Altschul et al. 1990) was performed, and all sequences were aligned using ClustalW (Thompson et al. 1994). A neighbor-joining tree of the aligned sequences was constructed according to the Jukes-Cantor model for calculating distance matrixes using MEGA V2.1 (Kumar et al. 2001). The neighbor-joining tree was bootstrapped 1000×. All sites including gaps in the sequence alignment were excluded pairwise in the phylogenetic analysis. I determined the taxonomic affiliation of each clone by comparing clones to their nearest phylogenetic neighbor within the neighbor-joining tree. I defined ribotypes as those sequences sharing 98% sequence identity with each other (Hongoh et al. 2003). The taxonomic assignment was confirmed at an 80% confidence level using the naïve Bayesian rRNA classifier algorithm on the RDP website (Cole et al. 2005). Sequence identity matrices were constructed and general manipulation

of the sequence data was accomplished using BIOEDIT V7.0 (Hall 1999). The sequence data from this study appear in the GenBank, EMBL, and DDBJ sequence databases as accession numbers DQ009673–DQ009714.

The diversity of the gut microbiota was analyzed by rarefaction analysis (Heck et al. 1975), using the software Analytic Rarefaction 1.3 (Holland 2006). Rarefaction analysis attempts to estimate the number of species (and place confidence levels on diversity) that would have been found had a smaller number of individuals been sampled. In addition, I calculated the species coverage (the estimated percentage of the total species samples) as described by Good (1953). The Chao1 nonparametric richness estimator (Chao 1987) was also calculated using the software package EstimateS (Colwell 2005). The Chao1 estimator provided a minimum estimate of the bacterial diversity in the termite gut. Estimation was also conducted using the estimator provided by Curtis et al. (2002) using the program Diversity Calculator v. 53, which is distributed online by the authors (http://people.civil.gla.ac.uk/~sloan/). Using this method, the diversity of the termite gut bacteria is estimated using the values N_T, N_{max}, and N_{min}, where N_T is the total number of individuals in the community, N_{max} is the number of individuals in the most abundant species, and N_{min} is the number of individuals in the least abundant species. I assumed N_{min} was 1. For N_T, I used the population estimations provided by Schultz and Breznak (1978) for the gut bacteria of *R. flavipes*. The total count of bacteria was estimated to be 10^{10} cells ml^{-1} gut fluid or 3×10^6 cells gut^{-1}. This method provides the maximum possible diversity of microorganisms in a community (Curtis et al. 2002).

ARDRA experiment. A total of 512 clones were analyzed by ARDRA (162 from PPA, 186 from PPB, 81 from BF, and 83 from PT). The 16S rRNA gene plasmid inserts were amplified by PCR from each site using *Taq* DNA polymerase (Promega) and a GeneAmp 9700 PCR System. Using a sterilized toothpick, I placed a small amount of each selected bacterial colony from the clone libraries into a 0.2 ml PCR tube filled with 50 μl of PCR reaction cocktail [1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton® X-100), 2 mM $MgCl_2$, 200 μM of each dNTP, 36.3 μl of ddH_20, and 0.5 μM of each primer (M13F and M13R)]. In order to burst the bacterial cell walls and release the plasmids, the resultant mixture was briefly mixed and incubated for 10 min at 94ºC in the thermocycler. At this point I quickly added 1.0 U *Taq* to the reaction cocktail and began the PCR cycling regime. The PCR cycling regime was as follows: 25 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 4 min, and a final elongation step at 72°C for 10 min.

The PCR products were then individually digested with the four-base cutting restriction enzyme *HhaI* (5'…GCGˇC…3') (Promega). Each digestion was performed in 1.5 ml reaction tubes as follows. Each 20 μl reaction contained 7.3 μl sterile water, 2 μl RE 10× buffer C (Promega), 0.2 μl acetylated BSA (Promega), 10 μl DNA, and 0.5 μl restriction enzyme. The resultant digestion mixture was incubated at 37°C for 2 hr, 4 μl of 6× loading dye was added (Promega), and the sample was stored in a -20°C freezer until needed for analysis.

I visualized restriction digest fragments on 2.5% NuSieve® GTG® Agarose gels (Cambrex) in 1× TBE buffer. Each gel was run for 1.5 hr at 100V. I then analyzed each gel on a Bio-Rad Molecular Imager Gel Doc XR System and with Quantity One 1-D

analysis software (Bio-Rad). Bands sizes were quantified for each lane by comparing them to a standard 50 bp DNA Ladder (BioLabs).

The diversity of the ARDRA profiles was analyzed using the same analyses described above for the sequencing data. I first computed the coverage as described by Good (1953). I also analyzed the data using rarefaction analysis (Heck et al. 1975) and calculated the estimators described by Chao (1987) and Curtis et al. (2002).

The ARDRA patterns from this study were compared to digitally digested termite gut bacteria 16S rRNA gene sequences gleaned from public sequence databases (i.e. GenBank) and from the 16S rRNA gene sequences from this study. I digested the 16S rRNA gene sequences using the TACG Restriction Mapping Tool found at http://biotools.umassmed.edu/tacg/WWWtacg.php. Digitally and experimentally derived ARDRA profiles were then compared by eye and the resultant matches were recorded.

Results and Discussion

16S rRNA gene sequences. I analyzed a total of 42 clone sequences, from which 33 distinct ribotypes were found (Fig. 3.1). No obvious chimeric artifacts were detected using the Check-Chimera Program on the Ribosomal Database Project web site (Cole et al. 2005). All 42 sequences, therefore, were used in the phylogenetic analysis.

Phylogenetic analysis of all sequences showed that the clones corresponded to a diverse range of members of the domain *Bacteria*. All sequences grouped into one of six major bacterial phyla: *Proteobacteria*, *Spirochaetes*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and the newly proposed "*Endomicrobia*" (previously termed *Termite Group I*) (Stingl et al. 2005) (Fig. 3.1). The phylogenetic analysis of 16S rRNA genes

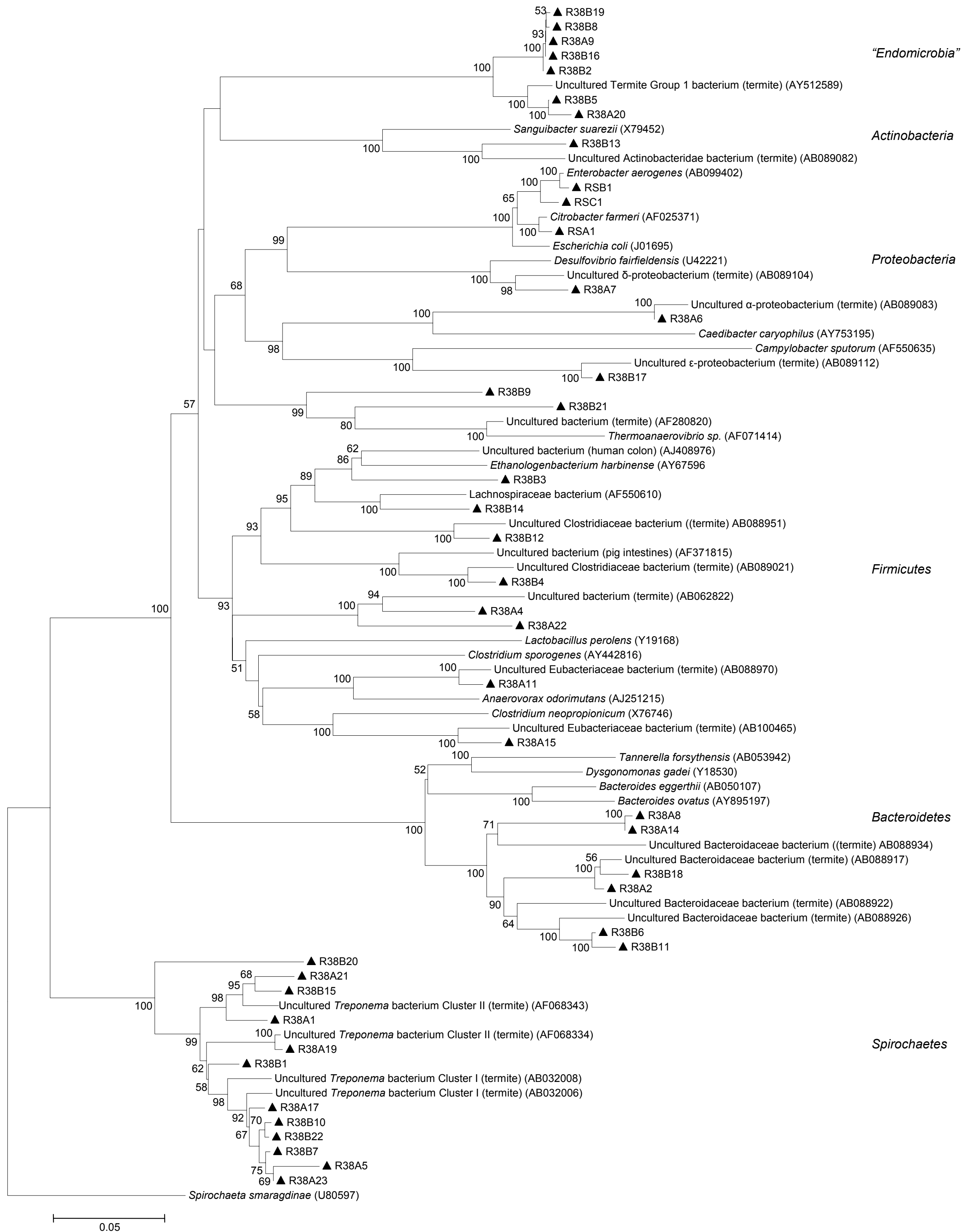

Fig. 3.1. Phylogenetic tree of 16S rRNA gene sequences of *R. flavipes* gut bacteria. The scale bar represents 0.05 substitutions per base position. Bootstrap values above 50 for 1000 resamplings (≥50% of the trees support the node) are shown for each node. These sequences correspond to positions 22–1400 in *E. coli.* Black triangles delineate samples sequenced in this study.

demonstrates that the gut bacterial microbiota of *R. flavipes* consists of many diverse species. It is noteworthy that of the 42 sequences analyzed in this study seven sequences (16%) (R38A8, R38A14, R38A22, R38B3, R38B11, R38B13, and R38B21) had less than 90% sequence similarity to previously characterized sequences (Altschul et al. 1990). It is possible that these seven sequences may represent undescribed bacterial genera. In addition, a large majority (92.9%) of the sequences that shared greater than 90% sequence similarity with other sequences in a BLASTN search (Altschul et al. 1990) did so only with uncultivated, and thus relatively uncharacterized, termite gut bacteria. It appears the bacteria from the termite gut share high sequence similarities with other bacteria from the termite gut but low sequence similarity with bacteria identified from other environments. This similarity in termite bacteria is evidence for the coevolution of the termite gut bacteria and their termite hosts. As suggested by Hongoh et al. (2003), more bacterial diversity data is needed from a variety of termite species in order to clarify the coevolutionary relationship between termites and their symbionts.

A rarefaction analysis of the sequence data resulted in a steep curve (Fig. 3.2). The steep curve shows that I did not sample but a small fraction of the ribotypes present in the gut. A much larger sample would be needed to capture a clear picture of the bacterial diversity present in the gut of *R. flavipes*. The homologous coverage, as calculated according to the method present by Good (1953), was only 28.6%. This result was interpreted to mean that at least 3.5× more bacterial ribotypes (~116) exist in the gut of *R. flavipes*. The Chao1 estimator (Chao 1987), which estimates a minimum number of existing ribotypes, was calculated to be 452.5. The methods described by Curtis et al. (2002) estimated there were 615 different ribotypes. These estimates corresponded

somewhat to the figure suggested by Hongoh et al. (2003). Hongoh et al. (2003) estimated, using the equation proposed by Curtis et al. (2002), that the gut of *R. speratus* contained ~700 different ribotypes. My data supports the expectation that closely related termite species would share similar patterns of bacterial diversity. In addition, these data suggest that a great proportion of the bacteria species occupying the gut of *R. flavipes* are still undescribed.

In the neighbor-joining tree, there is strong support for separating seven clones (Fig. 3.1; R38A9, R38A20, R38B2, R38B5, R38B8, R38B16, and R38B19) into a cluster corresponding with "*Endomicrobia*." These bacteria are cytoplasmic endosymbionts of the termite gut protozoa yet have been found only once before in *R. flavipes* (Stingl et al. 2005). My study confirmed the presence of "*Endomicrobia"* in *R. flavipes* and suggested, because it made up a high proportion (16.7%) of the clones, that "*Endomicrobia"* are found in high numbers within *R. flavipes*. In addition, further subgroups were found to exist within the "*Endomicrobia*" cluster. Clones R38A20 and

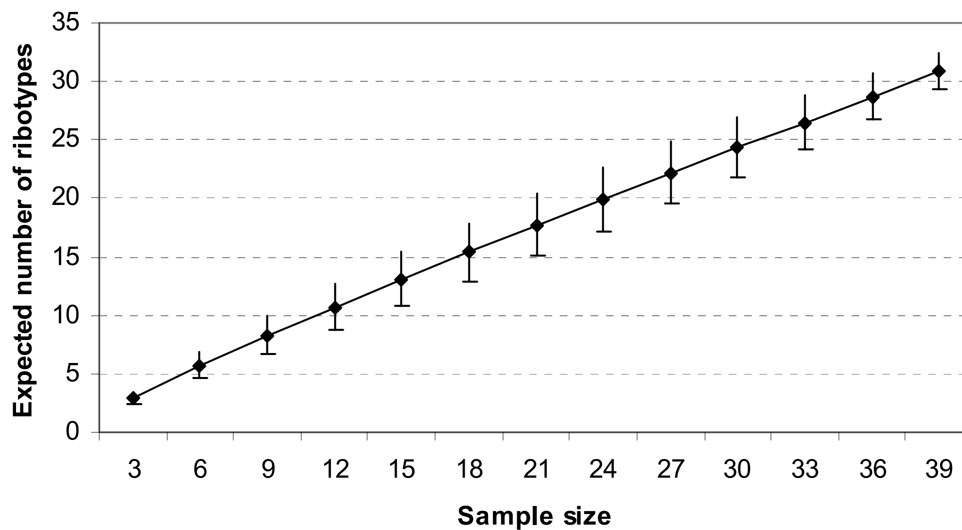

Fig. 3.2. Rarefaction curve for 16S rRNA gene sequences. The error bars represent the 95% confidence interval of the expected number of ribotypes for each sample size.

R38B5 form the first subgroup, sharing 98.3% sequence identity (Fig. 3.1). The clones in the second subgroup (R38A9, R38B2, R38B8, R38B16, and R38B19) share 99.5–99.8% sequence identity but no more than 94.1% sequence identity with clones in the first subgroup. Thus the "*Endomicrobia*" appeared to separate into two distinct ribotypes. Both subgroups were distantly related to all other clones sequenced in this study. Since the "*Endomicrobia*" are endosymbionts of the protozoa (Stingl et al. 2005), these two distinct clusters may correspond to the endosymbiotic bacteria present in two of the three common species of protozoa, *Trichonympha agilis*, *Pyrsonympha vertens*, and *Dinenympha gracilis* (Belitz and Waller 1998). If this idea is true, a more in-depth sampling of "*Endomicrobia"* in *R. flavipes* may result in the presence of three or more phyloge netic clusters corresponding to the three main species of protozoa.

Spirochaetes accounted for 29% (12 out of 42) of all clones (Fig. 3.1). A BLASTN (Altschul et al. 1990) search of GenBank revealed that the spirochete sequences (R38A1, R38A5, R38A17, R38A19, R38A21, R38A23, R38B1, R38B7, R38B10, R38B15, R38B20, and R38B22) shared high sequence identity with previous submissions that had been classified as *Treponema* spp. Based on high bootstrap values and branch depths, spirochete clones clustered with one of four main groups (Fig. 3.1). R38B20 represented a single example of the most basal group. The second group included clones R38A1, R38A21, and R38B15, and shared a relatively high sequence similarity to the Cluster II *Treponema* bacterium referenced as AF068343 in GenBank (Lilburn et al. 1999). The third group, including R38A19 and R38B1, formed an ambiguous cluster, due to low bootstrap values, with the Cluster II *Treponema* bacterium referenced as AF068334 (Lilburn et al. 1999). The final group included the remaining

six spirochete clones. High bootstrap values clustered this group closely with two Cluster I *Treponema* bacteria (AB032008 and AB032006) (Lilburn et al. 1999). I assigned all spirochete sequences, with the exception of R38B20, to the genus *Treponema* (Ohkuma et al. 1999, Hongoh et al. 2003, Noda et al. 2003) based on comparisons with the reference sequences obtained in GenBank. Because of its apparent phylogenetic distance from the other *Treponema* clones, I classified R38B20 as a member of *Spirochaeta*. The genus *Treponema* includes species that have been identified as obligate symbionts of the protozoa in the termite gut (Berchtold and Konig 1996, Paster et al. 1996). Found in very high numbers, these symbiotic spirochetes are attached to the outer cell surface of the protozoa and provide flagellate locomotion for the protozoa (Wenzel et al. 2003). The numerical abundance in this study of sequences corresponding to the genus *Treponema* supports previous findings that the genus *Treponema* performs an important role in the gut of *R. flavipes* (Lilburn et al. 1999).

Six clones were assigned to the phylum *Proteobacteria*. Clone R38A6 (Fig. 3.1) shared a deep branch and a 100% bootstrap value with a reference α-proteobacterium (AB089083) (Hongoh et al. 2003) and showed a low level of sequence identity with all other sequences. Clones RSA1, RSC1, and RSB1 grouped with the enteric bacteria within the γ subdivision of *Proteobacteria* (Fig. 3.1). RSA1 shared a 98.9% sequence similarity with *Citrobacter farmeri*, while both RSC1 and RSB1 shared a high amount of sequence identity (98.2% and 99.4% respectively) with *Enterobacter aerogenes*. Two closely related species, *Citrobacter freundii* and *Enterobacter agglomerans*, have both been implicated as nitrogen-fixers in termites (French et al. 1976, Potrikus and Breznak 1977). Thus RSA1, RSB1, and RSC1 may function as nitrogen fixers. One clone,

R38A7, clustered with the sulfur and sulfate reducing bacteria of the *δ-proteobacteria* subdivision. Another clone, R38B17, clustered with the *ε-proteobacteria* subdivision. Of the five major subdivisions within *Proteobacteria*, only a representative from *β-proteobacteria* was not found in my analysis.

With 10 clones, the phylum *Firmicutes* had the second largest representation (behind *Spirochaetes*) among the sequenced clones. Clones R38A4, R38A11, R38A15, R38A22, R38B3, R38B4, R38B9, R38B12, R38B14, and R38B21 clustered closely with GenBank reference sequences from *Firmicutes* (Fig. 3.1). Clones corresponding to *Firmicutes* also were taxonomically diverse. All clones were assigned to the order *Clostridiales* but were dispersed among several different families and genera. The functions of these diverse bacteria remain unknown.

The six sequences representing the phylum *Bacteroidetes* (R38A2, R38A8, R38A14, R38B6, R38B11, and R38B18) clustered with sequences that have previously only been described in termites (Fig. 3.1). I performed a BLAST search for sequences similar to the six *Bacteroidetes* sequences. The BLAST search did not return any sequences from non-termites origins with sequence identities greater than 88%. Thus it appears there is a diverse phylogenetic cluster of *Bacteroidetes* that is unique to termites. Further analysis is needed on this cluster of termite-specific bacteria, which I term the "Termite *Bacteroidetes* Group," to determine its exact phylogenetic position, the extent of its diversity, and its physiological role in termites. Further analysis, described in chapter five of this dissertation, demonstrated that these sequences were closely related to those *Bacteroidetes* previously described as endo- and ectosymbionts of the termite gut

protozoa (Noda et al. 2005, 2006). These bacteria, therefore, may be symbionts of the protozoa in *R. flavipes*.

Portions of the termite gut, especially the hindgut, of termites are highly anoxic (Bignell and Anderson 1980, Brune et al. 1995, Ebert and Brune 1997). Thus it would be expected that the bacteria inhabiting the gut would either be strict or facultative anaerobes. It is well established that the majority of members of certain bacterial taxa are anaerobes or microaerophilic, including the phylum "*Endomicrobia,*" the classes *Clostridia*, *Spirochaetes*, and *Bacteroidetes* and the orders *Enterobacteriales*, *Campylobacterales*, and *Desulfovibrionales* of *Proteobacteria* (Prescott et al. 2002). One could, therefore, reasonably expect to find representatives of known anaerobic taxa in high proportions. Indeed the data confirmed this expectation since members of anaerobic taxa made up 95.2% of all clones from my study. Only two clones, R38B13 (*Actinobacteria*) and R38A6 (*α-proteobacteria*) were classified into groups that were not considered either facultative or strict anaerobes. These two aerobic representatives could have originated from bacteria found in the more oxygen rich areas of the termite gut, such as the rectum. This study reconfirms, however, that anaerobic bacteria make up the majority of the bacteria found in the gut of *R. flavipes*.

A comparison between *R. flavipes* bacteria that had been previously identified via culturing techniques and the bacteria I characterized in this study resulted in striking differences. Schultz and Breznak (1978) conducted a study of the diversity of the gut microbiota of *R. flavipes* utilizing culture techniques. They found that the most abundant group of bacteria belonged to the genus *Streptococcus*. My sequence data did not include a single example of *Streptococcus*. In fact, no members of the class *Bacilli* were

detected. This difference in bacterial composition may have been due to the manner in which data were collected (i.e. culture vs. culture-independent techniques). It is also possible that members of *Streptococcus* are a numerical minority in the termite gut yet are more easily cultivable than their bacterial counterparts. DNA extraction and PCR amplification bias, or PCR primer mismatches may also offer plausible reasons for the absence of *Streptococcus* in my samples (Hongoh et al. 2003).

ARDRA Profiles. Because of the expensive nature of sequencing, I decided to do an additional analysis of the gut bacterial diversity in *R. flavipes* using ARDRA. I found 261 different ARDRA profiles out of the 512 clones I analyzed. The rarefaction analysis showed the slope beginning to decrease, suggesting that the ARDRA analysis, due to its greater sample size, captured a much larger amount of the diversity than did the sequencing analysis (Fig. 3.3). Good's (1963) coverage was calculated to be 66.4%, a likewise higher coverage than the sequencing analysis. Coverage of 66.4% could be interpreted to mean that at least 393 bacterial ribotypes exist in the gut of *R. flavipes*. The Chao1 estimator (Chao 1987) was 600.01. The Curtis estimator (Curtis et al. 2002) estimated 1,318 ribotypes. These estimates were extremely varied, yet the Chao1 estimator closely agreed with the estimation suggested by Hongoh et al. (2003). The wide variation between the estimates is likely due to two factors: differences in how the estimate is calculated and an insufficient sample size. The differences between the estimations provided by the sequencing analysis and the ARDRA analysis underscore the need for large samples when estimating the bacterial diversity in the termite gut. In addition, those utilizing these estimators should understand the differences between and limits of each estimator.

Many of the ARDRA profiles I analyzed closely corresponded to digitally digested profiles of known sequences (Table 3.1). By comparing the digitally digested sequences to the experimentally derived sequences, I was able to make theoretical assignments of the profiles to taxonomic groups. Many of the experimentally derived profiles did not match with any known sequences. This result suggests that a large proportion of the bacteria from *R. flavipes* is specific to *R. flavipes* and remains uncharacterized. A comparison of the taxonomic composition of the 16S rRNA gene sequences to the theoretical composition of the ARDRA profiles showed important differences (Fig. 3.4). Unlike the sequencing study, the ARDRA experiment found few "*Endomicrobia*" and a large number of individuals from the candidate phylum OP11. This result may be due to either (1) the small sample size of the sequencing study or (2) to the large number of unknowns in the ARDRA study. The "missing" "*Endomicrobia*" would likely be found if the unknown profiles were definitively characterized. More

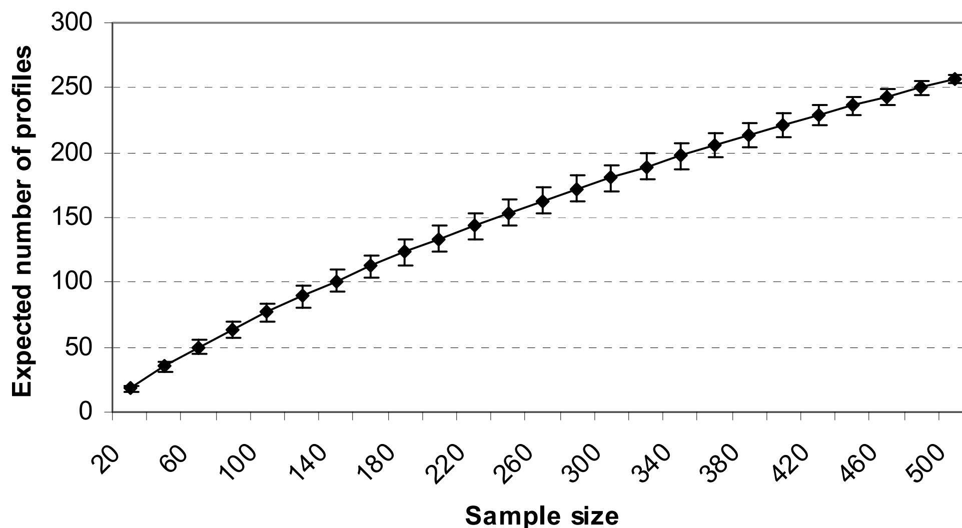

Fig. 3.3. Rarefaction curve for ARDRA profiles. The error bars represent the 95% confidence interval of the expected number of different profiles for each sample size.

sequencing data is needed against which ARDRA data can be compared.

For a complete picture of bacterial diversity in the termite gut, a concerted effort using both culture and culture-independent techniques is necessary. This study adds *R. flavipes* to the short list of termite species that have had their bacterial microbiota phylogenetically surveyed.

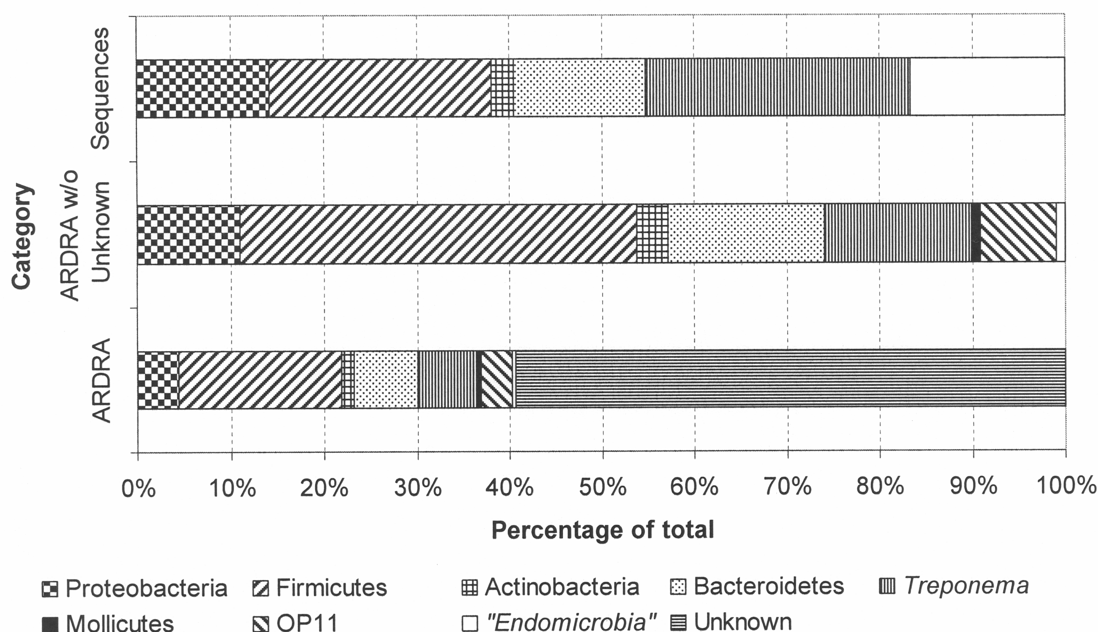

Fig. 3.4. Taxonomic proportions of the termite gut bacteria as measured using all of the ARDRA profiles (512 profiles), all of the ARDRA profiles except the "unknowns" (208 profiles), and 16S rRNA gene sequences from 42 samples.

Table 3.1. Assignment of clones from this study to known 16S rRNA bacterial sequences based on ARDRA profiles. The table matches the ARDRA profiles of clones from this study to digitally generated ARDRA profiles of known 16S rRNA gene sequences.

Profile name	Representative sequence (accession # | classification)	# clones	ARDRA profile (Band lengths)
AA	AB089105 | delta proteobacterium	2	277, 271, 255, 207, 166,165, 137, 91, 19, 2, 2
AE	AB089047 | Clostridiaceae bacterium	4	348, 256, 214, 184, 174, 165, 148, 70, 22
AM	AB088985 | Clostridiales bacterium	2	390, 347, 258, 225, 151, 128, 56
AN	AB089019 | Clostridiales bacterium	3	390, 347, 258, 184, 151, 112, 107, 2
AP	AB089057 | *Mycoplasma* sp.	1	394, 361, 280, 270, 250
AU	AB089070 | Actinobacteridae bacterium	2	408, 340, 262, 181, 178, 120, 63, 2
AW	AB089106 | delta proteobacterium	2	408, 397, 216, 206, 169, 135, 62
BA	AB089098 | alpha proteobacterium	3	410, 341, 259, 210, 184, 135
BB	AB089012 | Clostridiales bacterium	1	410, 345, 334, 301, 184
BN	AB089074 | Actinobacteridae bacterium	2	440, 408, 304, 249, 153
BR	AB089082 | Actinobacteridae bacterium	1	448, 408, 300, 263, 146
BT	AB088934 | Bacteroidaceae bacterium	4	454, 377, 247, 171, 149, 135, 31, 14
BV	AB089072 | Actinobacteridae bacterium	1	456, 411, 254, 157, 156, 116, 42
CI	AB088917 | Bacteroidaceae bacterium	10	485, 377, 247, 173, 149, 135, 14
CL	AB088931 | Bacteroidaceae bacterium	9	511, 375, 247, 178, 149, 113, 14
CO	AB089046 | Clostridiaceae bacterium	1	524, 404, 336, 189, 138, 2
CQ	AB088919 | Bacteroidaceae bacterium	2	525, 472, 247, 177, 163
CT	AB089108 | delta proteobacterium	5	531, 276, 273, 206, 138, 137, 31
CY	DQ0099675 | gamma proteobacterium	1	531, 451, 273, 197, 136
DB	AB088894 | *Treponema* sp.	3	534, 273, 249, 184, 149, 116, 70, 13
DD	AB088857 | *Treponema* sp.	11	534, 343, 249, 184, 150, 113, 13
DE	AB088875 | *Treponema* sp.	1	535, 259, 234, 184, 177, 116, 84
DJ	AB088898 | *Treponema* sp.	1	549, 415, 249, 149, 116, 88, 13, 10
DN	AB089112 | epsilon proteobacterium	2	567, 350, 265, 178, 106, 58, 42

Table 3.1 (Cont.). Assignment of clones from this study to known 16S rRNA bacterial sequences based on ARDRA profiles. The table matches the ARDRA profiles of clones from this study to digitally generated ARDRA profiles of known 16S rRNA gene sequences.

Profile name	Representative sequence (accession # | classification)	# clones	ARDRA profile (Band lengths)
DR	AB088882 | *Treponema* sp.	1	590, 389, 249, 149, 116, 82, 13
DT	AB089084 | alpha proteobacterium	1	590, 412, 207, 184, 139
DU	AB088867 | *Treponema* sp.	1	590, 471, 249, 149, 116, 13
EA	AB088943 | Bacteroidaceae bacterium	2	620, 375, 247, 166, 149, 14
EC	AB089016 | Clostridiales bacterium	1	621, 409, 345, 184
ED	AB089099 | alpha proteobacterium	4	621, 409, 346, 171
EE	AB089066 | Unknown bacterium	1	623, 507, 411, 27
EF	DQ009696 | Firmicutes bacterium	2	630, 348, 250, 184, 130, 24
EL	AB089100 | beta proteobacterium	3	651, 474, 409, 54
EM	AB089030 | Clostridiaceae bacterium	4	654, 348, 273, 184, 130
EN	AB089029 | Clostridiaceae bacterium	2	654, 403, 348, 184
ES	AB089063 | *Lactococcus* sp.	2	668, 527, 408
EU	AB089036 | Clostridiaceae bacterium	6	673, 475, 250, 153, 54
EZ	AB088973 | Eubacteriaceae bacterium	1	703, 457, 385, 25
FE	AB089073 | Actinobacteridae bacterium	1	706, 449, 263, 146
FG	AB088955 | Eubacteriaceae bacterium	2	711, 441, 409
FH	AB089040 | Clostridiaceae bacterium	1	711, 477, 405
FP	DQ009703 | *Treponema* sp.	1	718, 273, 248, 162, 120, 70
FW	AB089003 | Clostridiales bacterium	3	816, 408, 221, 108
FY	AB088903 | *Treponema* sp.	1	827, 248, 164, 149, 116, 70, 13
GB	AB088878 | *Treponema* sp.	4	842, 248, 149, 141, 116, 70, 13, 8
GC	AB088953 | Clostridiaceae bacterium	1	845, 403, 292
GI	AB089062 | *Enterococcus* sp.	1	872, 408, 297, 29
GK	AB088907 | *Treponema* sp.	3	889, 249, 164, 149, 116, 13, 8
GO	AB088996 | Eubacteriaceae bacterium	1	900, 406, 250
GP	AB088972 | Eubacteriaceae bacterium	2	901, 410, 270
GU	AB088913 | *Treponema* sp.	2	991, 249, 149, 116, 70, 13
GW	AB088929 | Bacteroidaceae bacterium	2	988, 241, 185, 169

Table 3.1 (Cont.). Assignment of clones from this study to known 16S rRNA bacterial sequences based on ARDRA profiles. The table matches the ARDRA profiles of clones from this study to digitally generated ARDRA profiles of known 16S rRNA gene sequences.

Profile name	Representative sequence (accession # | classification)	# clones	ARDRA profile (Band lengths)
GY	AB088947 | Bacteroidaceae bacterium	1	997, 247, 157, 149, 14
GZ	AB088924 | Bacteroidaceae bacterium	1	996, 247, 184, 149, 14
HA	AB088942 | Bacteroidaceae bacterium	1	998, 175, 149, 137, 110, 14
HB	AB089052 | "*Endomicrobia*" bacterium	1	1021, 408, 138
HC	AB088983 | Clostridiaceae bacterium	7	1035, 407, 136, 2
HD	AB088887 | *Treponema* sp.	1	1061, 177, 149, 116, 72, 13
HM	AB088876 | *Treponema* sp.	3	1091, 249, 149, 70, 13
HN	AB089114 | OP11 bacterium	4	1118, 394
HO	AB088951 | Clostridiaceae bacterium	3	1136, 407
HP	AB089125 | OP11 bacterium	13	1141, 394
HQ	AB089027 | Clostridiaceae bacterium	2	1157, 411
HS	DQ009685 | Firmicutes bacterium	1	1147, 272, 136
HW	AB089048 | "*Endomicrobia*" bacterium	1	1160, 408
HX	AB089064 | Unknown bacterium	2	1166, 410
HY	AB089058 | *Mycoplasma* sp.	1	1167, 243, 149
IA	AB088950 | Eubacteriaceae bacterium	1	1169, 223, 137, 50
IB	AB088968 | Clostridiaceae bacterium	3	1170, 402
ID	AB088966 | Clostridiaceae bacterium	32	1191, 408
MA-TJ	None	304	Varied

References Cited

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Bauer, S., A. Tholen, J. Overmann, and A. Brune. 2000. Characterization of abundance and diversity of lactic acid bacteria in the hindgut of wood- and soil-feeding termites by molecular and culture-dependent techniques. Arch. Microbiol. 173: 126-137.

Belitz, L. A., and D. A. Waller. 1998. Effect of temperature and termite starvation on phagocytosis by protozoan symbionts of the Eastern subterranean termite, *Reticulitermes flavipes* Kollar. Microbial Ecol. 36: 175-180.

Berchtold, M., and H. Konig. 1996. Phylogenetic analysis and *in situ* identification of uncultivated spirochetes from the hindgut of the termite *Mastotermes darwiniensis*. Syst. Appl. Microbiol. 19: 66-73.

Bignell, D. E., and J. M. Anderson. 1980. Determination of pH and oxygen status in the guts of lower and higher termites. J. Insect Physiol. 26: 183-188.

Brauman, A., J. Dore, P. Eggleton, D. Bignell, J. A. Breznak, and M. D. Kane. 2001. Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. FEMS Microbiol. Ecol. 35: 27-36.

Breznak, J. A. 2000. Phylogenetic diversity and physiology of termite gut spirochetes. Am. Zool. 40: 954-955.

Breznak, J. A., W. J. Brill, J. W. Mertins, and H. C. Coppel. 1973. Nitrogen fixation in termites. Nature 244: 577-580.

Breznak, J. A., and M. D. Kane. 1990. Microbial H_2/CO_2 acetogenesis in animal guts - nature and nutritional significance. FEMS Microbiol. Rev. 87: 309-313.

Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 75: 4801-4805.

Brune, A., D. Emerson, and J. A. Breznak. 1995. The termite gut microflora as an oxygen sink - microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. 61**:** 2681-2687.

Chao, A. 1987. Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43: 783-791.

Chu, S. 2005. Biology as a solution to physics problems. Plenary speech, Institute of Physics Conference: Physics 2005, 14 April 2005, Warwick, UK.

Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje. 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 33 (Database Issue)**:** D294-D296.

Colwell, R. K. 2005. EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5. User's Guide and application published at: http://purl.oclc.org/estimates.

Curtis, T. P., W. T. Sloan, and J. W. Scannell. 2002. Estimating prokaryote diversity and its limits. Proc. Natl. Acad. Sci. USA 99: 10494-10499.

Ebert, A., and A. Brune. 1997. Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite *Reticulitermes flavipes* (Kollar). Appl. Environ. Microbiol. 63: 4039-4046.

French, J. R., G. L. Turner, and J. F. Bradbury. 1976. Nitrogen fixation by bacteria from the hindgut of termites. J. Gen. Microbiol. 96**:** 202-206.

Good, I. J. 1953. The population frequencies of species and the estimation of population parameters. Biometrika 40: 237-264.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41**:** 95-98.

Heck, K.L., Jr., G. Van Belle, and D. Simberloff. 1975. Explicit calculation of the rarefaction diversity measurement and the determination of sufficient sample size. Ecology 56:1459-1461.

Holland, S. M. 2006. Analytic rarefaction user’s manual, version 1.3. Steven M. Holland, University of Georgia, GA.

Hongoh, Y., M. Ohkuma, and T. Kudo. 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol. Ecol. 44**:** 231-242.

Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, abd T. Kudo. **2005**. Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl. Environ. Microbiol. 71: 6590-6599.

Hongoh, Y. P., L. Ekpornprasit, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, N. Noparatnaraporn, and T. Kudo. 2006. Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. Mol. Ecol. 15: 505-516.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180: 4765-4774.

Keeling, P. J., N. Poulsen, and G. I. McFadden. 1998. Phylogenetic diversity of parabasalian symbionts from termites, including the phylogenetic position of *Pseudotrypanosoma* and *Trichonympha*. J. Eukaryot. Microbiol. 45**:** 643-650.

Kellenberger, E. 2001. Exploring the unknown: the silent revolution of microbiology. EMBO Rep. 2: 5-7.

Kudo, T., M. Ohkuma, S. Moriya, S. Noda, and K. Ohtoko. 1998. Molecular phylogenetic identification of the intestinal anaerobic microbial community in the hindgut of the termite, *Reticulitermes speratus*, without cultivation. Extremophiles 2**:** 155-161.

Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics 17**:** 1244-1245.

Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283**:** 686-689.

Lilburn, T. G., T. M. Schmidt, and J. A. Breznak. 1999. Phylogenetic diversity of termite gut spirochaetes. Environ. Microbiol. 1**:** 331-345.

Marchesi, J. R., T. Sato, A, J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64**:** 795-799.

Noda, S., M. Ohkuma, A. Yamada, Y. Hongoh, and T. Kudo. 2003. Phylogenetic position and in situ identification of ectosymbiotic spirochetes on protists in the termite gut. Appl. Environ. Microbiol. 69**:** 625-633.

Noda, S., T. Iida, O. Kitade, H. Nakajima, T. Kudo, and M. Ohkuma. 2005. Endosymbiotic *Bacteroidales* bacteria of the flagellated protist *Pseudotrichonympha grassii* in the gut of the termite *Coptotermes formosanus*. Appl. Environ. Microbiol. 71: 8811-8817.

Noda, S., T. Inoue, Y. Hongoh, M. Kawai, C. Nalepa, C. Vongkaluang, T. Kudo, and M. Ohkuma. 2006. Identification and characterization of ectosymbionts of distinct lineages in *Bacteroidales* attached to flagellated protists in the gut of termites and a wood-feeding cockroach. Environ. Microbiol. 8:11-20.

Ohkuma, M., T. Iida, and T. Kudo. 1999. Phylogenetic relationships of symbiotic spirochetes in the gut of diverse termites. FEMS Microbiol. Lett. 181**:** 123-129.

Ohkuma, M., and T. Kudo. 1998. Phylogenetic analysis of the symbiotic intestinal microflora of the termite *Cryptotermes domesticus*. FEMS Microbiol. Lett. 164**:** 389-395.

Ohkuma, M., and T. Kudo. 1996. Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. Appl. Environ. Microbiol. 62**:** 461-468.

Osborn, A. M., E. R. Moore, and K. N. Timmis. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2**:** 39-50.

Paster, B. J., F. E. Dewhirst, S. M. Cooke, V. Fussing, L. K. Poulsen, and J. A. Breznak. 1996. Phylogeny of not-yet-cultured spirochetes from termite guts. Appl. Environ. Microbiol. 62**:** 347-352.

Potrikus, C. J., and J. A. Breznak. 1977. Nitrogen-fixing *Enterobacter agglomerans* isolated from guts of wood-eating termites. Appl. Environ. Microbiol. 33**:** 392-399.

Prescott, L. M., J. P. Harley, and D. A. Klein. 2002. Microbiology. McGraw-Hill Companies, Inc., New York, N.Y.

Scheffrahn, R. H., and N. Y. Su. 1994. Keys to soldier and winged adult termites (Isoptera) of Florida. Fla. Entomol. 77**:** 460-474.

Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune. 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites. Appl. Environ. Microbiol. 69: 6018-6024.

Schultz, J. E., and J. A. Breznak. 1978. Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. Appl. Environ. Microbiol. 35**:** 930-936.

Sharma, R., R. Ranjan, R. K. Kapardar, and A. Grover. 2005. 'Unculturable' bacterial diversity: an untapped resource. Curr. Sci. 89: 72-77.

Shinzato, N., M. Muramatsu, T. Matsui, and Y. Watanabe. **2005**. Molecular phylogenetic diversity of the bacterial community in the gut of the termite *Coptotermes formosanus*. Biosci. Biotechnol. Biochem. 69: 1145-1155.

Slaytor, M. 1992. Cellulose digestion in termites and cockroaches - what role do symbionts play? Comp. Biochem. Physiol. B 103**:** 775-784.

Stingl, U., R. Radek, H. Yang, and A. Brune. 2005. "Endomicrobia": cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. Appl. Environ. Microbiol. 71**:** 1473-1479.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22**:** 4673-4680.

To, L. P., L. Margulis, D. Chase, and W. L. Nutting. 1980. The symbiotic microbial community of the Sonoran Desert termite: *Pterotermes occidentis*. Biosystems 13**:** 109-137.

Weidner, S., W. Arnold, and A. Puhler. 1996. Diversity of uncultured microorganisms associated with the seagrass *Halophila stipulacea* estimated by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. 62**:** 766-771.

Wenzel, M., R. Radek, G. Brugerolle, and H. Konig. 2003. Identification of the ectosymbiotic bacteria of *Mixotricha paradoxa* involved in movement symbiosis. Eur. J. Protistol. 39**:** 11-23

Chapter Four
Effects of Food Source on the Gut Microbiota of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae)

Introduction

The termite gut is a complex micro-ecosystem consisting of a diverse array of protozoa, bacteria, archaea, and fungi. The relationship between the termite host and its gut symbionts is a form of obligatory mutualism. The microorganisms serve a variety of important functions for the host termite, including cellulase production (Slaytor 1992), acetogenesis (Breznak and Kane 1990, Leadbetter et al. 1999), and nitrogen acquisition (Breznak et al. 1973). In return, the termite gut provides the symbionts with an optimal environment for growth and reproduction by supplying a steady influx of nutrients.

It has been suggested that another possible function of the termite gut microorganisms is a role in termite intra- and intercolony recognition. Adams (1991) made several interesting statements in his discussion of termite interaction:

> "Workers of (*Microcerotermes*) *arboreus* recognized unfamiliar relatives and distinguished degrees of similarity among unfamiliar kin. These results demonstrate that there is a strong inherited component to the cues used in nest-mate recognition…
>
> …the possibility of nongenetic inheritance cannot be ruled out. Odors may be carried from the source nest by alate reproductives and transferred directly to their offspring…Symbiotic bacteria used in digestion of cellulose are also transferred from parent to offspring, but there is no evidence that these affect surface pheromones."

The three key points in Adam's (1991) discussion were (1) that nestmate recognition cues are inherited, (2) that non-genetic inheritance needs to be considered, and (3) that symbiotic bacteria are a nongenetically inherited factor.

Alates, by way of trophallaxis, inoculate their newly formed colonies with symbiotic microorganisms, providing a nongenetically inherited component to their

offspring (Snyder 1948). As the colony grows, the job of faunating the newly hatched termites is performed by the workers. In addition, the workers constantly participate in trophallaxis amongst themselves (Snyder 1948). Thus, due to the continual exchange of digestive fluids within a colony, all members of a colony should have a similar gut microbiota originally inherited from the founding alates.

As previously mentioned, Adams (1991) stated that no evidence existed that the termite microbiota performs any role in constructing nestmate recognition surface pheromones. Matsuura (2001) found, however, that intestinal bacteria in *R. speratus* mediated nestmate recognition. In his study, Matsuura (2001) used antibiotics and bacterial extracts to show that bacteria influence the degree of aggression demonstrated between and within colonies of *R. speratus*. Therefore, the chemicals given off by bacteria in the gut and excreted in termite feces might be the chemical odors termites use in nestmate recognition (Matsuura 2001).

The findings by Matsuura (2001) are particularly interesting in light of a study by Shelton and Grace (1997), which concluded that an unknown environmental influence might perform an important role in a “multiple component system for intercolony kin recognition” in *C. formosanus.* Shelton and Grace (1997) found that no antagonism existed between laboratory colonies that were “exogenously similar, but genetically different.” Since nestmate recognition is not correlated with genetic relatedness, doubt is cast on the hypothesis that the termite’s DNA is the heritable factor involved in termite nestmate recognition.

It is possible that the bacteria described by Matsuura (2001) are the environmental influence suggested by Shelton and Grace (1997) and the heritable factor described by

Adams (1991) that regulates nestmate recognition. This idea becomes more compelling as one discovers that the termite gut microbiota is heavily influenced by environmental factors such as temperature (Belitz and Waller 1998), oxygen level (Tholen et al. 1997), and food source (Mannesmann 1972, Cook and Gold 2000). If it can then be assumed that if bacterially derived, endogenous chemicals are the cues termites use in nestmate recognition, then changes to the environment will change the gut bacterial microbiota and in turn the nestmate recognition cues. Thus bacteria mediated nestmate recognition would tie together seemingly contradictory studies implicating either environmental factors (Shelton and Grace 1997) or heritable factors (Adams 1991).

This study seeks to clarify the role of bacteria in nestmate recognition. Using behavioral assays, manipulation of the gut microbiota, and genetic characterization of the manipulated microbiota, I tried to find evidence for the idea that the bacteria in the termite gut perform a role in nestmate recognition. In particular, I looked for evidence that manipulating the termite diet of nestmates would (1) induce aggression and (2) change the gut microbiota. If a change in aggression coincided with a change in the microbiota, a link between aggression (recognition) and the gut microbiota could be deduced.

Materials and Methods

Termite collection, identification, and assay preparation. Three colonies of *R. flavipes* (PP-1, PP-2, and BF-1) were collected from fallen dead wood in Montgomery County, Virginia, United States. Termite soldiers were used to identify the colonies as *R. flavipes* (Scheffrahn and Su 1994).

The colonies were extracted from their wood source and placed into separate plastic Petri dishes with five 0.5 cm × 2.0 cm × 8.0 cm slats of white pine. The colonies acclimated to their new food source for 16 days. After acclimation, the colonies were each split into two subcolonies. One subcolony (labeled colony A) was left on the original white pine food source and the other (labeled colony B) was placed into a Petri dish with Recruit II™ bait matrix (Dow AgroSciences) that had no active ingredient. The subcolonies were allowed to feed at room temperature (~72º C) for 15 days.

Aggression assays. For all colonies, termites from the two subcolonies were assayed for changes in nestmate recognition due to changes in diet. Changes in nestmate recognition were measured using an aggression assay. The level of aggression demonstrated between two subcolonies (A vs. B) was determined using the protocol established by Clément (1986). The aggression levels of intracolony pairings fed on different food sources were compared to aggression levels of intracolony pairings fed on the same food source (white pine). The subcolony pairings took place in Petri dishes (25 mm × 150 mm; LabTek). Petri dishes were lined with Whatman 44 Ashless circle filter papers (125 mm) moistened with 2 ml of tap water. Upon completion of setup, termite pairings were maintained at room temperature for 24 hrs.

The aggression study involved 480 termites in 24 pairings. For each pairing, 20 termite workers from each of the two subcolonies were counted out and separated using an aspirator. At initiation, all 40 termites were placed simultaneously onto opposite ends of a piece of moistened filter paper in a Petri dish. Each pairing was replicated five times. After 24 hrs each Petri dish was opened and the total number of dead and injured

termites was counted. The antagonism level (AG) of each pairing was measured using the following index:

$$AG = 2.5\left(\overline{M} + \overline{m} / 2\right)$$

where "$\overline{M}$ is the mean number of dead termites in the five Petri dishes and $\overline{m}$ the mean number of injured workers" (Clément 1986). Individuals missing either both antennae or more than one leg were counted as moribund. Termites found with one antenna or a missing leg were labeled as "injured". From this formula, variation in aggression between two subcolonies could range from 0 (no aggression) to 100 (all workers dead). We followed the suggestion by Clément (1986) defining high levels of aggression as all indexes greater than or equal to 25.

The data were analyzed using a two-tailed *t*-test to determine significant differences in aggression behavior between control colonies (from the original white pine acclimated colony) and the subcolonies in question. *P*-values less than 0.05 were used to indicate significance (SAS Institute 2003).

ARDRA Analysis. I genetically analyzed the gut microbiota of colony PP-1 throughout the aggression assays looking for changes in the gut community structure. Thirty workers (termed sample PP-1Orig) were removed from the originally acclimated white pine colony for genetic analysis as described below. These 30 workers provided baseline data against which to compare future data. After the 15 days of feeding on their respective food source, 30 workers were also removed from both of the split subcolonies (termed PP-1Recr for the Recruit II™ sample and PP-1Post for the white pine sample) and their gut microbiota genetically analyzed as described below.

Amplified rDNA restriction analysis (ARDRA) was used to analyze for differences in the gut microbiota of the sub-samples. I first extracted the gut bacterial DNA from the 30 termites from each sub-sample. For each DNA extraction, the 30 termite workers were washed and their guts were excised using sterilized forceps. I placed the 30 guts in a single 1.5 ml microcentrifuge tube filled with 200 μl of PBS buffer solution [130 mM NaCl, 10 mM sodium phosphate buffer (pH 7.2)] and gently crushed the guts using a sterilized pestle. Gut cells were harvested by centrifugation (5000 × *g* for 10 min), decanting the supernatant, and resuspending the pellet in 180 μl of enzymatic lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100). Lysozyme (20 mg ml^{-1}; Fisher Scientific) was mixed into the sample of gut cells and incubated for 30 min at 37°C. After incubation, I added 25 μl of proteinase K and 200 μl of buffer AL (Qiagen) to the gut sample and the mixture was vortexed and incubated for 30 min at 70°C. DNA was then extracted using the Qiagen DNeasy Tissue Kit (Qiagen), starting at step 4 of the "Purification of Total DNA from Animal Tissue" protocol that accompanies the extraction kit.

The 16S rRNA gene was amplified by PCR from the extracted DNA using *Taq* DNA polymerase (Promega) and a GeneAmp 9700 PCR System. Each 40 μl PCR reaction contained 0.8 μl of the DNA extract, 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton® X-100), 2 mM $MgCl_2$, 200 μM of each dNTP, 1.6 U *Taq*, 14.08 μl of ddH_20, and 2 μM of each primer. The bacteria-specific PCR primers I used were 41F (Weidner et al. 1996) and 1389R (Marchesi et al. 1998, Osborn et al. 2000). The PCR cycling regime was as follows: 2 min of the initial denaturation at 95°C followed by 24 cycles at 95°C for 30 s, 50°C for 1 min, and 74°C for 4 min, and a final elongation

step at 74°C for 10 min. The correct PCR product size (~1.4 kb) was confirmed by electrophoresis on a 1.0 % low-melting-point agarose gel (Amresco). I completed PCR clean-up with a QIAquick PCR Purification Kit (Qiagen) following manufacturer protocols.

Using the TOPO TA Cloning Kit (Invitrogen), PCR products were then cloned into pCR®II-TOPO® cloning vectors, and DH5α-T1 strain *Escherichia coli* cells were transformed with the vectors following manufacturer protocols. Clone libraries were then established on selective LB agar plates. I used blue-white screening to select for cells containing plasmids with the intended insert.

Sixty 16S rRNA gene plasmid inserts were amplified by PCR from each sample using *Taq* DNA polymerase (Promega) and a GeneAmp 9700 PCR System. Using a sterilized toothpick, I placed a small amount of each selected clone into a 0.2 ml PCR tube filled with 50 µl of PCR reaction cocktail [1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton® X-100), 2 mM $MgCl_2$, 200 µM of each dNTP, 36.3 µl of ddH_20, and 0.5 µM of each primer (M13F and M13R)]. In order to burst the bacterial cell walls and release the plasmids, the resultant mixture was briefly mixed and incubated for 10 min at 94ºC in the thermocycler. At this point I immediately added 1.0 U *Taq* to the reaction cocktail and began the PCR cycling regime. The PCR cycling regime was as follows: 25 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 4 min, and a final elongation step at 72°C for 10 min.

The PCR products were then individually digested with the four-base cutting restriction enzyme *HhaI* (5'…GCGˇC…3') (Promega). Each digestion was performed in 1.5 ml reaction tubes as follows. Each 20 µl reaction contained 7.3 µl sterile water, 2 µl

RE 10 × buffer C (Promega), 0.2 μl acetylated BSA (Promega), 10 μl DNA, and 0.5 μl restriction enzyme. The resultant digestion mixture was incubated at 37°C for 2 hr, 4 μl of 6× loading dye was added (Promega), and the sample was stored in a -20°C freezer until analysis.

Restriction digest fragments were visualized on 2.5% NuSieve® GTG® Agarose gels (Cambrex) in 1× TBE buffer. Each gel was run for 1.5 hr at 100V. I then analyzed each gel on a Bio-Rad Molecular Imager Gel Doc XR System and with Quantity One 1-D analysis software (Bio-Rad). Bands sizes were quantified for each lane by comparing them to a standard 50 bp DNA Ladder (BioLabs).

The main purpose of the analysis was to determine if ARDRA could distinguish between the gut microbiota of termite colonies feeding on different food sources. To do this, I analyzed the data using several different indices of community similarity. These indices determined if the proportion of ARDRA profiles shared between the two subcolonies (PP-1Recr and PP-1Post) was different than the proportions of ARDRA profiles shared between the subcolonies and the original colony (PP-1Orig). For each comparison, I calculated the Bray-Curtis sample dissimilarity index (Magurran 1988, 2004) and the classic Jaccard sample similarity index (Chao et al. 2005). For the Bray-Curtis index, the values range from 0 to 1, with 0 equating to perfect similarity and 1 equating to no similarity. For the Jaccard index, the values also range from 0 to 1, but 0 is equated with no similarity and 1 with perfect similarity. These similarity indices were calculated using the program EstimateS (Colwell 2005).

I also analyzed the data using a modified version of the analysis described in Heyndrickx et al. (1996). A similarity matrix was calculated by comparing the combined

ARDRA profile of each gut population with one another. For each sample comparison, the Dice similarity coefficient (S_D) (Nei and Li 1979) was calculated, according to the formula,

$$S_D = 2\frac{n_{AB}}{(n_A + n_B)}$$

with n_{AB} the number of ARDRA profiles common between population A and population B, n_A the number of ARDRA profiles in population A, and n_B the number of profiles in population B. The values range from 0 to 1, with 0 equating to no similarity and 1 equating to perfect similarity.

The diversity of the ARDRA profiles was determined by rarefaction analysis (Heck et al. 1975), using the Analytic Rarefaction 1.3 software program (Holland 2006). Rarefaction analysis attempts to estimate the number of species that would have been found had a smaller number of individuals been sampled.

Results and Discussion

The purpose of my first evaluation was to determine if food source affected termite nestmate recognition. Pairings of nestmates fed different food sources yielded low aggression indices (AG) in all cases (Table 4.1). The aggression levels were comparable to the control pairings. Clément (1986) defined high aggression levels to be 25 or higher. The highest AG was displayed by colony BF-1, which had an AG of 0.5 (Table 4.1). Since the control pairing for BF-1 demonstrated an aggression level of 1.13 and since the result was significantly lower than the threshold suggested by Clément (1986), an AG of 0.5 was interpreted as essentially no aggression. Thus none of the pairings displayed any aggression toward each other. Nor was any statistical difference

Table 4.1. Data showing the aggression index (AG) for pairings of termites. The first three pairings show data for intracolony pairings of individuals fed on two different food sources (A&B). The last three show the AG of intercolony pairings. The control AG is the aggression index of intracolony pairings fed on the same food source (white pine).

Pairing	Pairing AG	Control AG	*P*-value
PP-1 A vs. B	0.15	0.13	0.89
PP-2 A vs. B	0.10	0.13	0.85
BF-1 A vs. B	0.50	1.13	0.09
PP-1 vs. PP-2	0.25	n/a	n/a
PP-1 vs. BF-1	0.75	n/a	n/a
PP-2 vs. BF-1	0.00	n/a	n/a

found between the aggression in pairings of nestmates fed on different food sources and aggression in pairings of nestmates fed on the same food source.

I also tested if any aggression was displayed between intercolony pairings. No aggression was demonstrated between non-nestmates (Table 4.1). Though the original colonies were all found at distances greater than 1 km from one another, the termites did not show overt intercolony aggressive behavior. This result may explain why nestmates that fed on different food sources did not demonstrate aggressive behavior towards one another. If *R. flavipes* colonies generally do not demonstrate intercolony aggression, it is unlikely they would display aggression against nestmates fed on different food sources.

A lack of aggression between different termite colonies has been reported before in the literature. Fisher and Gold (2003) reported a lack of aggression in many colonies of *R. flavipes* in Texas. Fisher and Gold (2003) found that intercolony aggression was variable within *R. flavipes*. Several colonies, including one pairing of colonies 49 km apart, showed no aggression while other colonies showed strong aggressive behavior. Bulmer and Traniello (2002) reported a similar finding. A lack of aggression between

intercolony pairings in my study may be due to the small sample size. A larger sample of colonies of *R. flavipes* in Virginia may yield intercolony aggression.

The lack of aggression between nestmates fed on different food sources (Table 4.1) may be evidence that food source is not a factor in determining nestmate recognition cues. Food source cannot, however, be completely ruled out. Florane et al. (2004) found that diet affected the intercolony aggression in the Formosan subterranean termite, *Coptotermes formosanus*. In my study, I only looked at the effects of a single artificial food source, Recruit II™, on nestmate recognition. A broader sampling of termite colonies fed multiple types of artificial and natural food sources is needed.

I was also not able to determine if the change in food source affected the gut microbiota. I expected to find a greater degree of similarity in the gut bacterial ARDRA profiles of the control pairing (PP-1Orig vs. PP-1Post) than the test pairing (PP-1Orig vs. PP-1Recr). This result was expected because both subcolonies in the control pairing were fed on the same food source, and the subcolonies in the test pairing were fed on different food sources. The percentage, however, of the ARDRA profiles shared was similar for both the control and test pairings (Table 4.2). There were, in addition, no major differences in the indices of similarity between the control pairing and the test pairing. In fact, the different indices gave conflicting results. Both the Dice and Bray-Curtis (dis)similarity indices suggested that the ARDRA profiles for the test pairing were more similar than the ARDRA profiles for the control pairing (Table 4.2). The Jaccard similarity index, however, indicated that the ARDRA profiles of the test pairing were less similar than the ARDRA profiles of the control pairing. Thus the similarity indices suggested that either food source does not affect the termite gut microbiota or that the

Table 4.2. Similarity/dissimilarity indices comparing the termite gut bacteria ARDRA profiles of three samples (PP-1Orig, PP1-Recr, and PP-1Post) fed on different food sources.

Pairing	% profiles shared	Dice	Bray-Curtis	Jaccard
PP-1Orig vs. PP-1Recr	10.1	0.149	0.198	0.101
PP-1Orig vs. PP-1Post	10.3	0.143	0.222	0.103
PP-1Recr vs. PP-1Post	9.9	0.144	0.208	0.099

protocols followed in this study were insufficient for detecting changes in the gut microbiota due to manipulation of the termite's food source.

It is possible that the ability to detect changes in the gut microbiota due to changes in the termite host's food source were compromised by a small ARDRA profile sample size. My study was limited due to a lack of time and financial resources to a small ARDRA profile sample size. I was only able to perform ~60 ARDRA profiles for each subcolony. The small sample size limited my ability to capture a true picture of the termite gut bacterial community diversity. Because of the preponderance of ARDRA

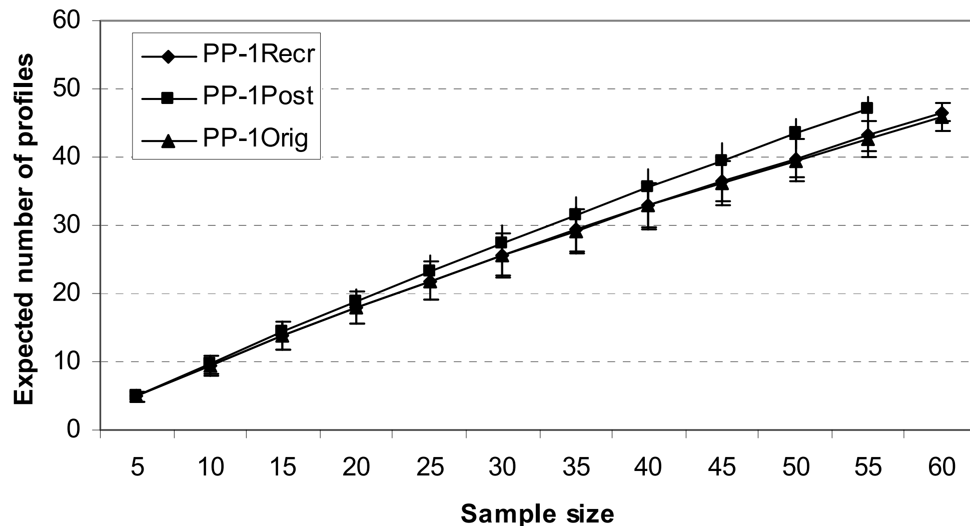

Fig. 4.1. Rarefaction curves of the gut bacterial ARDRA profiles for PP-1Recr, PP-1Post, and PP-1Orig. The error bars represent 95% confidence intervals for the expected number of profiles.

profiles that were found only once, the small sample size limited the resolution power of the similarity indices. A rarefaction analysis of the data supported the idea that a small sample size limited the resolution power of the similarity indices (Fig. 4.1). The rarefaction analysis showed that I sampled only a small minority of the bacterial species present in the guts of the termites I sampled. If the majority of the species present in a particular environment are found in a diversity survey, it is expected that the rarefaction curves for that particular diversity survey would plateau (Heck et al. 1975). If the majority of the species have not been found, the rarefaction curve would instead follow a steep angle and lack a plateau (Heck et al. 1975). In this current study, the rarefaction curves of the ARDRA profiles for each subcolony followed a steep angle for the entirety of the graph and did not plateau (Fig. 4.1). Thus a much larger set of ARDRA profiles would be needed to detect changes in the gut bacterial community in relation to changes in food source.

The fact that the similarity indices did not provide evidence for change in the bacterial community as a result of change in the termite food source does not rule out that change does occur when food sources change. In fact, it is very likely food source does affect the termite gut bacterial community. Carter et al. (1981) found that the termite gut protozoan community structure of *C. formosanus* is affected by the type of wood the termite is feeding on. Thus it is likely that a more in-depth study that includes a larger ARDRA profile sample size would detect the effects of termite food source on the gut bacterial community.

References Cited

Adams, E. S. 1991. Nest-mate recognition based on heritable odors in the termite *Microtermes arboreus*. Proc. Natl. Acad. Sci. 88: 2031-2034.

Belitz, L. A., and D. A. Waller. 1998. Effect of temperature and termite starvation on phagocytosis by protozoan symbionts of the Eastern subterranean termite *Reticulitermes flavipes*. Microbial Ecol. 36: 175-180.

Breznak, J. A., W. J. Brill, J. W. Mertins, and H. C. Coppel. 1973. Nitrogen fixation in termites. Nature 244: 577-580.

Breznak, J. A., and M. D. Kane. 1990. Microbial H_2/CO_2 acetogenesis in animal guts - nature and nutritional significance. FEMS Microbiol. Rev. 87: 309-313.

Bulmer, M. S., and J. F. A. Traniello. 2002. Lack of aggression and spatial association of colony members in Reticulitermes flavipes. J. Insect Behav. 15:121-126.

Carter, F. L., J. K. Mauldin, and N. M. Rich. 1981. Protozoan populations of *Coptotermes formosanus* Shiraki exposed to heartwood samples of 21 American species. Material un Organismen 16: 29-38.

Chao, A., R. L. Chazdon, R. K. Colwell, and T.–J. Shen. 2005. A new statistical approach to assessing compositional similarity based on incidence and abundance data. Ecol. Lett. 8: 148-159.

Clément, J. L. 1986. Open and closed societies in *Reticulitermes* termites (Isoptera: Rhinotermitidae): geographic and seasonal variations. Sociobiology 11: 311-323.

Colwell, R. K. 2005. EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5. User's Guide and application published at: http://purl.oclc.org/estimates.

Cook, T. J., and R. E. Gold. 2000. Effects of different cellulose sources on the structure of the hindgut flagellate community in *Reticulitermes virginicus* (Isoptera: Rhinotermitidae). Sociobiology 35: 119-130.

Fisher, M. L., and R. E. Gold. 2003. Intercolony aggression in *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Sociobiology 42: 651-661.

Florane, C. B., J. M. Bland, C. Husseneder, A. K. Raina. 2004. Diet-mediated inter-colonial aggression in the Formosan subterranean termite *Coptotermes formosanus*. J. Chem. Ecol. 30: 2559-2574.

Heck, K.L., Jr., G. Van Belle, and D. Simberloff. 1975. Explicit calculation of the rarefaction diversity measurement and the determination of sufficient sample size. Ecology 56:1459-1461.

Heyndrickx, M., L. Vauterin, P. Vandamme, K. Kersters, and P. De Vos. 1996. Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. J. Microbiol. Methods 26: 247-259.

Holland, S. M. 2006. Analytic rarefaction user's manual, version 1.3. Steven M. Holland, University of Georgia, GA.

Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283**:** 686-689.

Magurran, A. E. 1988. Ecological diversity and its measurement. Princeton University Press, Princeton, NJ.

Magurran, A. E. 2004. Measuring biological diversity. Blackwell Publishing, Boston, MA.

Mannesmann, R. 1972. Relationship between different wood species as a termite food source and the reproduction rate of termite symbionts. Z. Ang. Ent. 72: 116-128.

Marchesi, J. R., T. Sato, A, J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64:795-799.

Matsuura, K. 2001. Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos 92: 20-26.

Nei, M., and W.-H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269-5273.

Osborn, A. M., E. R. Moore, and K. N. Timmis. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2:39-50.

SAS Institute. 2003. SAS 9.1.3 help and documentation. SAS Institute, Cary, NC.

Scheffrahn, R. H., and N.–Y. Su. 1994. Key to soldier and winged adult termites (Isoptera) of Florida. Fla. Entomol. 77: 460-474.

Shelton, T. G., and J. K. Grace. 1997. Suggestion of an environmental influence on intercolony agonism of Formosan subterranean termites (Isoptera: Rhinotermitidae). Environ. Entomol. 26: 632-637.

Slaytor, M. 1992. Cellulose digestion in termites and cockroaches - what role do symbionts play? Comp. Biochem. Physiol. B 103**:** 775-784.

Tholen, A., B. Schink, and A. Brune. 1997. The gut microflora of *Reticulitermes flavipes*, its relation to oxygen, and evidence for oxygen-dependent acetogenesis by the most abundant *Enterococcus* sp. FEMS Microbiol. Ecol. 24: 137-149.

Weidner, S., W. Arnold, and A. Puhler. 1996. Diversity of uncultured microorganisms associated with the seagrass *Halophila stipulacea* estimated by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. 62:766-771.

Chapter Five
Analysis of the bacterial diversity between species of termites: evidences for coevolution

Introduction

The termite gut is a complex micro-ecosystem consisting of a diverse array of microorganisms, including protozoa, bacteria, archaea, and fungi. The gut microorganisms and their termite hosts are examples of a form of obligatory mutualism. The microorganisms serve a variety of important functions for the host termite, including cellulase production (Slaytor 1992), acetogenesis (Breznak and Kane 1990, Leadbetter et al. 1999), and nitrogen acquisition (Breznak et al. 1973). In return, the termite gut provides an optimal environment for growth and reproduction, and the microorganisms receive a steady influx of nutrients.

Several species of termites have had the diversity of their gut microbiota surveyed, including species of both higher (e.g. *Microcerotermes* spp. by Hongoh et al. 2005) and lower termites (e.g. *R. speratus* by Hongoh et al. 2003). In chapter three of this dissertation, I did a small survey of the gut bacteria of *Reticulitermes flavipes*. I noticed that many of the ribotypes I discovered clustered closely with bacterial ribotypes described from other species of termites. In addition, many of the ribotypes I found were phylogenetically distant from bacterial ribotypes originating from non-termite sources. These clustering patterns seemed to suggest that the termite gut bacteria and their hosts share an intimate coevolutionary relationship.

Hongoh et al. (2005) described a study in which they found evidence for coevolution of the gut microbiota and their termite hosts. In the study, the authors compared the 16S rRNA gene sequences and terminal restriction fragment length

polymorphism (T-RFLP) patterns of four species within the genus *Reticulitermes* and four species of *Microcerotermes*. Hongoh et al. (2005) utilized both inter- and intraspecific comparisons to look for patterns of coevolution. They found that a substantial number of the 16S rRNA gene ribotypes clustered into monophyletic groups corresponding to the originating termite genera. In fact, only one ribotype was found to be shared by the two genera. In another study, Schmitt-Wagner et al. (2003) used T-RFLP data to demonstrate similarity in the gut microbiota between three termite species in the genus *Cubitermes*. In an analysis of different gut sections, they found moderate to high similarity between the three species for each gut section. These studies suggest that the termite gut microbiota and their termite hosts have coevolved.

In this study, I used the *R. flavipes* gut bacterial 16S rRNA gene sequence data collected in chapter three of this dissertation and all known termite gut bacteria 16S rRNA gene sequences from public sequence databases (i.e. GenBank) to look for patterns of coevolution between termites and their gut bacteria. In particular, I focused on looking for evidence supporting coevolution at two different taxonomic levels within termites, the order and genus levels.

Materials and Methods

Termite collection and identification. *R. flavipes* were collected from a single colony in fallen dead wood from Prince George County, Virginia, United States. In order to avoid the possible effects of laboratory storage on the termite gut microbiota, I only used freshly collected termites for all analyses. Termites from the soldier caste were used to identify the colony as *R. flavipes* (Scheffrahn and Su 1994).

DNA extraction, PCR, and cloning. Preliminary to the DNA extraction, 30 termite workers were washed and their guts were excised using sterilized forceps. I placed the 30 guts in a single 1.5 ml microcentrifuge tube filled with 200 μl of PBS buffer solution [130 mM NaCl, 10 mM sodium phosphate buffer (pH 7.2)] and gently crushed the guts using a sterilized pestle. Gut cells were harvested by centrifugation (5000×*g* for 10 min), decanting the supernatant, and resuspending the pellet in 180 μl of enzymatic lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100). Lysozyme (20 mg ml^{-1}; Fisher Scientific) was mixed into the sample of gut cells and incubated for 30 min at 37°C. After incubation, I added 25 μl of proteinase K and 200 μl of buffer AL (Qiagen) to the gut sample and the mixture was vortexed and incubated for 30 min at 70°C. DNA was then extracted using the Qiagen DNeasy Tissue Kit (Qiagen), starting at step 4 of the "Purification of Total DNA from Animal Tissue" protocol that accompanies the extraction kit.

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) from the extracted DNA using *Taq* DNA polymerase (Promega) and a GeneAmp 9700 PCR System. The 40 μl PCR reaction contained 0.8 μl of the DNA extract, 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton® X-100), 2 mM $MgCl_2$, 200 μM of each dNTP, 1.6 U *Taq*, 14.08 μl of ddH_20, and 2 μM of each primer. The bacteria-specific PCR primers I used were 41F (Weidner et al. 1996) and 1389R (Marchesi et al. 1998, Osborn et al. 2000). The PCR cycling regime was as follows: 2 min of the initial denaturation at 95°C followed by 24 cycles at 95°C for 30 s, 50°C for 1 min, and 74°C for 4 min, and a final elongation step at 74°C for 10 min. The correct PCR product size (~1.4 kb) was confirmed by electrophoresis on a 1.0 % low-melting-point agarose gel

(Amresco). I completed PCR clean-up with a QIAquick PCR Purification Kit (Qiagen) following manufacturer protocols.

Using the TOPO TA Cloning Kit (Invitrogen), the PCR products were cloned into pCR®II-TOPO® cloning vectors, and DH5α-T1 strain *Escherichia coli* cells were transformed with the vectors following manufacturer protocols. Clone libraries were then established on selective LB agar plates. I used blue-white screening to select for cells containing plasmids with the intended insert.

Miniprep cleanup and 16S rRNA sequencing. Selected cells were grown overnight in ampicillin selective LB broth. Miniprep cleanup of resulting colonies was accomplished using the QIAprep Spin Miniprep Kit (Qiagen) following manufacturer protocols.

Forty-two clones were sequenced. I performed DNA sequencing at the Virginia Bioinformatics Institute (VBI) Core Laboratory Facility (Blacksburg, Virginia), and at the GeneLab/BioMed facility within the Louisiana State University School of Veterinary Medicine (Baton Rouge, Louisiana). Approximately 1200-1400 bp [corresponding to positions 22-1400 in *E. coli* (J01695)(Brosius et al. 1978)] of the clones were sequenced using the Big-Dye Terminator Cycle Sequencing Kit (Perkin-Elmer), an ABI 370 or 3700 genetic analyzer, and primers T7, 41F, and 1389R. I checked all sequences for chimeric artifacts using the Check-Chimera program on the Ribosomal Database Project (RDP) web site (Cole et al. 2005).

Analysis. I included 1,450 sequences in the phylogenetic analysis. The sequences included those from this study (42 total sequences), all known termite gut bacteria 16S rRNA gene sequences in GenBank (1165 total sequences), and 243

reference sequences found using BLASTN V2.2 (Altschul et al. 1990). Gut bacterial sequences originating from many different termite species were used, including: *R. flavipes*, *R. speratus*, *R. santonensis*, *Coptotermes formosanus*, *Cryptotermes secundus*, *Cryptotermes domesticus*, *Hodotermes mossambicus*, *Hodotermes sjoestedti*, *Kalotermes flavicollis*, *Mastotermes darwiniensis*, *Neotermes cubanus*, *Neotermes koshunensis*, *Schedorhinotermes lamanianus*, *Zootermopsis nevadensis*, two uncharacterized *Microcerotermes* sp., an uncharacterized *Reticulitermes* sp., and uncharacterized *Archotermopsis* sp., and *Macrotermes gilvus*. Only positions corresponding to positions 22-1400 in *E. coli* were used in the analysis. All sequences were aligned using ClustalW (Thompson et al. 1994). A neighbor-joining tree of the aligned sequences was constructed according to the Jukes-Cantor model for calculating distance matrixes using MEGA V2.1 (Kumar et al. 2001). The neighbor-joining tree was bootstrapped 1000×. All sites including gaps in the sequence alignment were excluded pairwise in the phylogenetic analysis. I determined the taxonomic affiliation of each clone by comparing clones to their nearest phylogenetic neighbor within the neighbor-joining tree. For evidence of coevolution, I searched for termite gut bacterial sequences in the neighbor-joining tree that clustered tightly with other sequences originating from bacteria in the gut of termites. General manipulation of the sequence data was accomplished using BIOEDIT V7.0 (Hall 1999). The sequence data from this study appear in the GenBank, EMBL, and DDBJ sequence databases as accession numbers DQ009673–DQ009714.

Results and Discussion

In this study, I aimed to examine whether the gut bacteria in termites coevolved with their termite hosts. In particular, I looked for evidence of termite specific bacterial lineages. My study found strong evidence of coevolution at both the bacterial genus and order taxonomic levels. The majority of the termite gut bacterial sequences clustered closely with other ribotypes from termite guts, though the sequences originated from distantly related termites. Because of the extensiveness of the phylogenetic analysis (1,450 sequences across at least 15 phyla), I decided to focus on the four bacterial phyla (*Bacteroidetes*, *Actinobacteria*, *Spirochaetes*, and "*Endomicrobia*") that demonstrated the clearest evidence for coevolution.

Many of the termite-derived bacterial ribotypes formed monophyletic clusters specific to the termite order Isoptera (termed "termite cluster"). A good example of a monophyletic cluster within *Bacteroidetes* corresponding to order Isoptera is found in Fig. 5.1. Fifty-two ribotypes from distantly related termite species formed a well-defined "termite cluster." It is interesting to note that four of the ribotypes within the "termite cluster" (designated with an *) were previously described as being either ecto- or endosymbionts of the gut symbiotic protozoa (Noda et al. 2005, 2006). The protozoa, in the lower termites, produce cellulases, and are thus indispensable to the termite (Slaytor 1992). Because of the tight symbiotic relationship between the cellulase producing protozoa and their host termites, it is possible that the two organisms coevolved (Hafner and Nadler 1988, Page 1994). The clustering of symbionts by host taxonomic level in phylogenetic analyses is a diagnostic characteristic of coevolution (Hafner and Nadler 1988). Thus my results may be indicative of coevolution of the *Bacteroidetes* "termite

cluster" and termites. Since only four of the ribotypes within the "termite cluster" have been definitively associated with the protozoa (Fig. 5.1) and some of the ribotypes within the cluster originated from higher termites (e.g. *Microcerotermes*), which do not have cellulase producing protozoa (Slaytor 1992), further study of this cluster is needed to determine which of the members of the "termite cluster" are bacterial symbionts of the termite gut symbiotic protozoa. Similar order-level "termite clusters" were found in *Actinobacteria* (Fig. 5.2). The *Actinobacteria*, however, have so far not been implicated in symbiotic relationships with the gut protozoa. If coevolution has occurred between the *Actinobacteria* and their termite hosts, different selecting factors must be involved other than a tight relationship with the symbiotic protozoa.

In addition to clustering at the order level, I found many bacterial ribotypes clustering at the genus level within Isoptera (Figs. 5.1, 5.3, and 5.4). When analyzing at the termite genus level, I observed a difference in tree topologies between the bacterial phyla. For most trees (Figs. 5.1 and 5.3), the termite genus-level clusters appeared to have gone through fewer speciation events than those clusters in the *Treponema* tree (Fig. 5.4). The higher level of speciation in *Treponema* is suggested by the presence of a greater number of clusters for each termite genus. For example, whereas the *Bacteroidetes* exhibited only three major *Reticulitermes* clusters ("major" being defined as having three or more ribotypes in the cluster) (Fig. 5.1), the *Treponema* exhibited 11 major *Reticulitermes* clusters that were dispersed between clusters from other termite genera (Fig. 5.4). Similar findings were evident for the termite genus *Microcerotermes*. The higher number of clusters may indicate that the *Treponema* speciated earlier than the *Bacteroidetes*. In fact, the highly dispersed clusters of *Treponema* may suggest that the

Treponema went through multiple speciation events both prior to and after an ancient termite speciation event. Alternatively, the tight clustering of the *Bacteroidetes* relative to their corresponding termite genera suggests that the *Bacteroidetes* underwent the majority of their speciation after each termite speciation event.

The coevolution of the termite gut microbiota and their termite hosts has also been evidenced by studies of the protozoan and archaeal gut symbionts. Inoue et al. (2000) showed that the majority of the protozoa associated with termites are unique to the lower termites and wood-feeding cockroaches in the genus *Cryptocercus*. Some archaeal symbionts have also been shown to be specific to termite guts (Tokura et al. 2000, Friedrich et al. 2001, Donovan et al. 2004). Combined with the results of this study, it thus appears that the majority of the identified termite gut microbiota is unique to termites and has coevolved with their termite hosts.

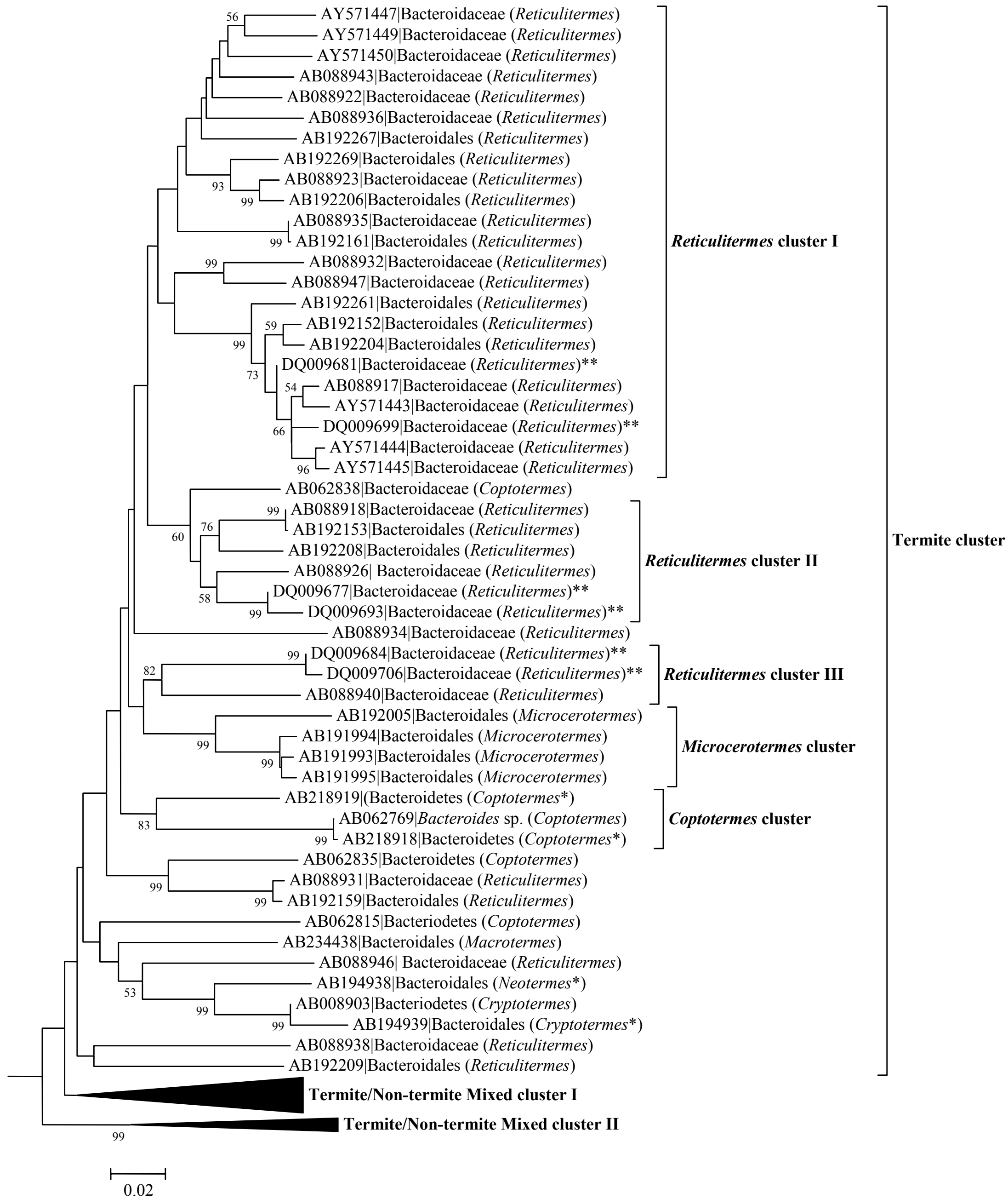

Fig. 5.1. Neighbor-joining phylogenetic tree showing the relationships of 16S rRNA ribotypes of termite gut bacteria affiliated with the phylum *Bacteroidetes*. Only bootstrap values ≥50 for 1000 resamplings are shown. Only positions corresponding to positions 22-1400 in *E. coli* are used in the analysis. The scale bar represents 0.02 substitutions per base position. The labels for each sample are given as "accession #|ribotype taxonomy (corresponding termite genus)." Cluster names are shown in bold. Those ribotypes with known affiliation to symbiotic protozoa are labeled with an *. Those ribotypes from this study are labeled with a **.

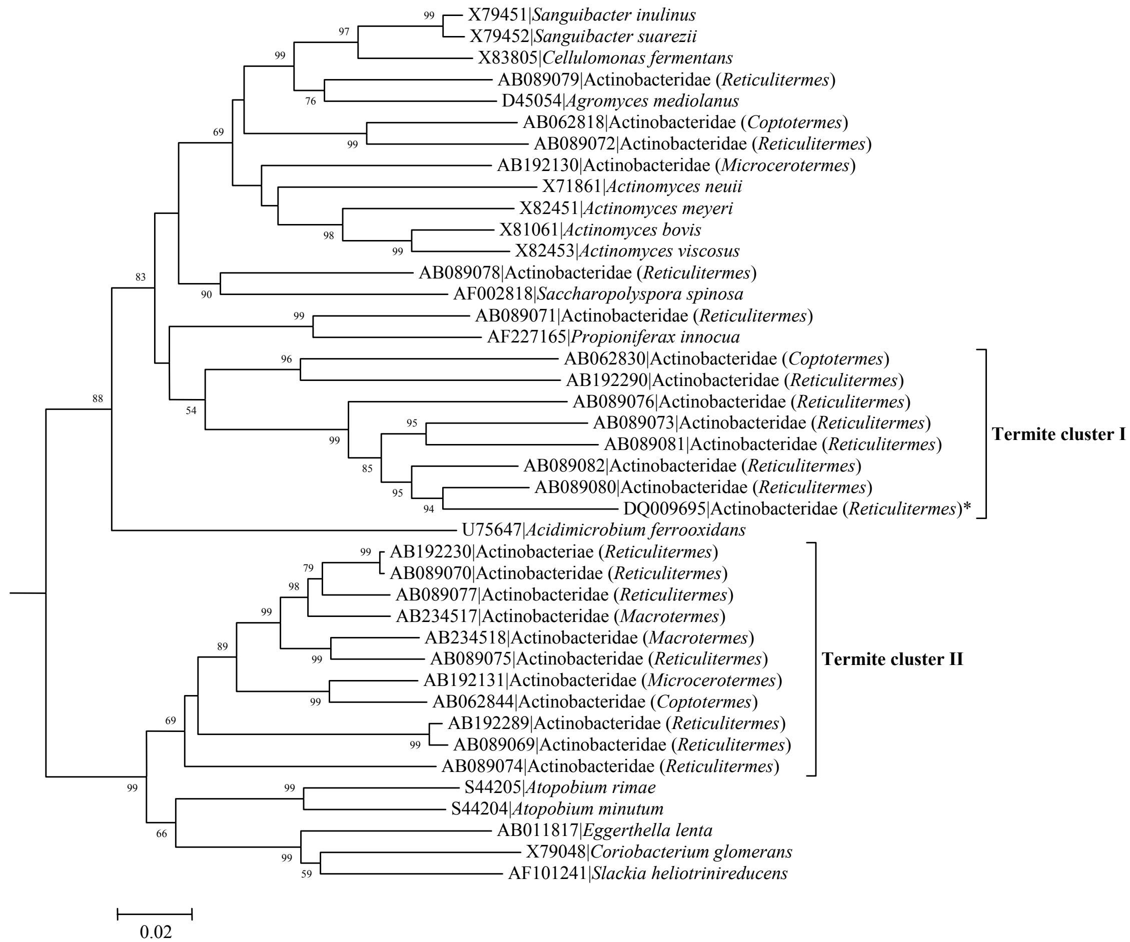

Fig. 5.2. Neighbor-joining phylogenetic tree showing the relationships of 16S rRNA ribotypes of termite gut bacteria affiliated with the phylum *Actinobacteria*. Only bootstrap values ≥50 for 1000 resamplings are shown. Only positions corresponding to positions 22-1400 in *E. coli* are used in the analysis. The scale bar represents 0.02 substitutions per base position. The labels for each sample are given as "accession #|ribotype taxonomy (corresponding termite genus)." Cluster names are shown to the right side in bold. Those ribotypes from this study are labeled with a *.

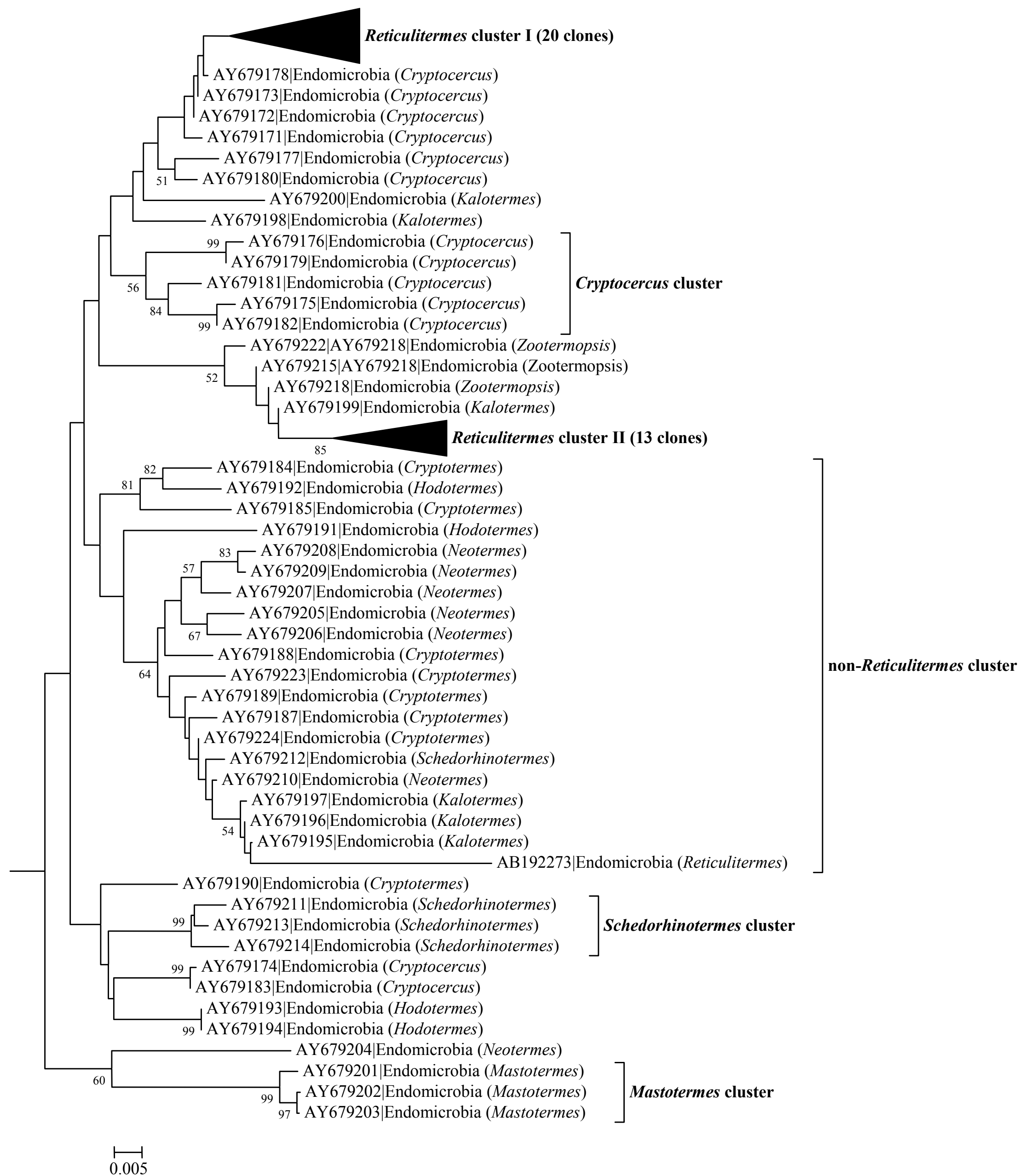

Fig. 5.3. Neighbor-joining phylogenetic tree showing the relationships of 16S rRNA ribotypes of termite gut bacteria affiliated with the proposed phylum "*Endomicrobia*." Only bootstrap values ≥50 for 1000 resamplings are shown. Only positions corresponding to positions 22-1400 in *E. coli* are used in the analysis. The scale bar represents 0.005 substitutions per base position. The labels for each sample are given as "accession #|ribotype taxonomy (corresponding termite genus)." Cluster names are shown in bold.

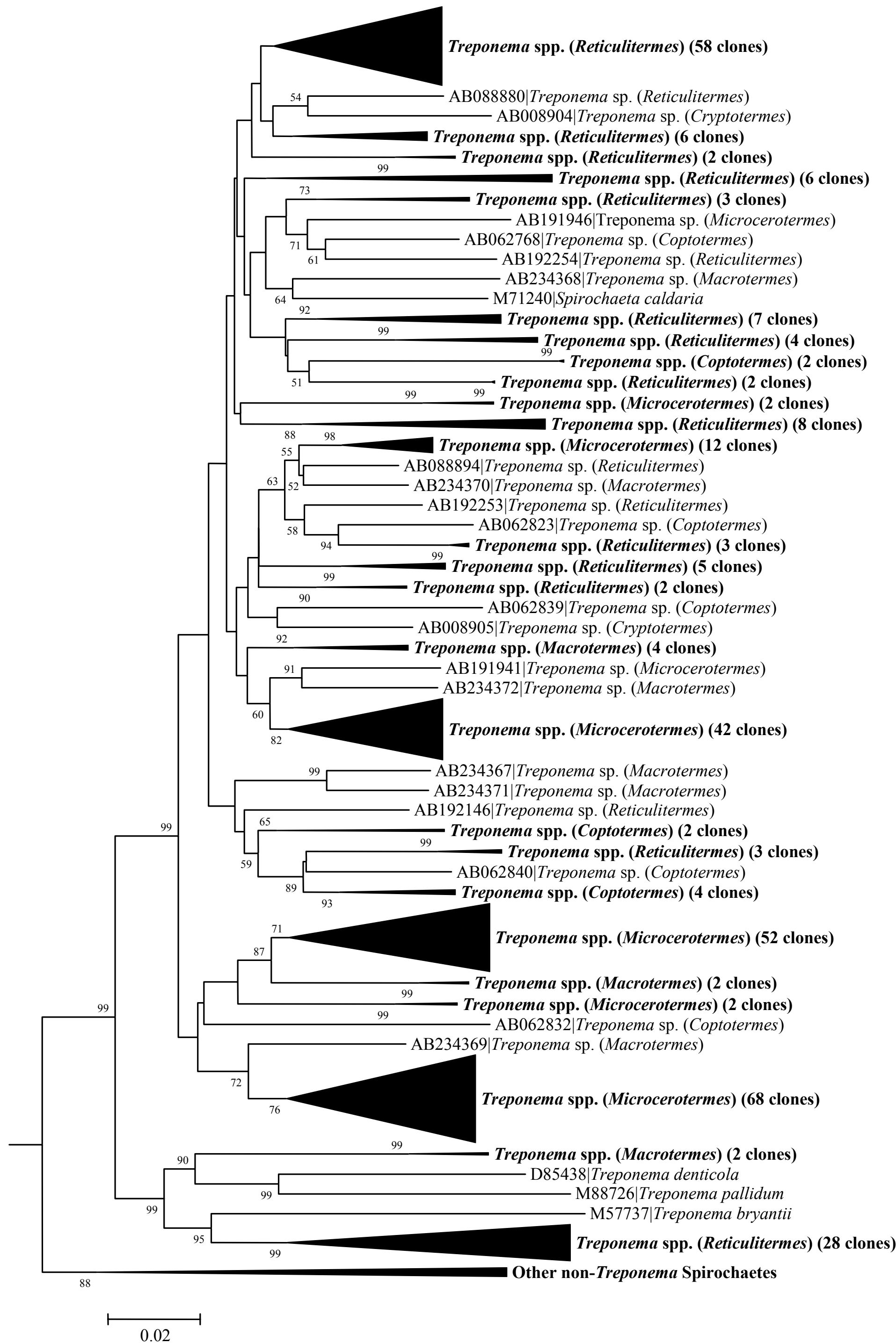

Fig. 5.4. Neighbor-joining phylogenetic tree showing the relationships of 16S rRNA ribotypes of termite gut bacteria affiliated with the phylum *Spirochaetes*. Only bootstrap values ≥50 for 1000 resamplings are shown. Only positions corresponding to positions 22-1400 in *E. coli* are used in the analysis. The scale bar represents 0.02 substitutions per base position. The labels for each sample are given as “accession #|ribotype taxonomy (corresponding termite genus).” Cluster names are shown in bold.

References Cited

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Breznak, J. A., W. J. Brill, J. W. Mertins, and H. C. Coppel. 1973. Nitrogen fixation in termites. Nature 244: 577-580.

Breznak, J. A., and M. D. Kane. 1990. Microbial H_2/CO_2 acetogenesis in animal guts - nature and nutritional significance. FEMS Microbiol. Rev. 87: 309-313.

Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 75: 4801-4805.

Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje. 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 33 (Database Issue)**:** D294-D296.

Donovan, S. E., K. J. Purdy, M. D. Kane, and P. Eggleton. 2004. Comparison of *Euryarchaea* strains in the guts and food-soil of the soil-feeding termite *Cubitermes fungifaber* across different soil types. Appl. Environ. Microbiol. 70: 3884-3892.

Friedrich, M. W., D. Schmitt-Wagner, T. Lueders, and A. Brune. 2001. Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl. Environ. Microbiol. 67: 4880-4890.

Hafner, M. S., and S. A. Nadler. 1988. Phylogenetic trees support the coevolution of parasites and their hosts. Nature 332: 258-259.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41**:** 95-98.

Hongoh, Y., M. Ohkuma, and T. Kudo. 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol. Ecol. 44**:** 231-242.

Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, abd T. Kudo. **2005**. Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl. Environ. Microbiol. 71: 6590-6599.

Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics 17**:** 1244-1245.

Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283**:** 686-689.

Marchesi, J. R., T. Sato, A, J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64**:** 795-799.

Noda, S., T. Iida, O. Kitade, H. Nakajima, T. Kudo, and M. Ohkuma. 2005. Endosymbiotic *Bacteroidales* bacteria of the flagellated protist *Pseudotrichonympha grassii* in the gut of the termite *Coptotermes formosanus*. Appl. Environ. Microbiol. 71: 8811-8817.

Noda, S., T. Inoue, Y. Hongoh, M. Kawai, C. Nalepa, C. Vongkaluang, T. Kudo, and M. Ohkuma. 2006. Identification and characterization of ectosymbionts of distinct lineages in *Bacteroidales* attached to flagellated protists in the gut of termites and a wood-feeding cockroach. Environ. Microbiol. 8:11-20.

Osborn, A. M., E. R. Moore, and K. N. Timmis. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2**:** 39-50.

Page, R. 1994. Parallel phylogenies: reconstructing the history of host-parasite assemblages. Cladistics 10: 155-175.

Scheffrahn, R. H., and N. Y. Su. 1994. Keys to soldier and winged adult termites (Isoptera) of Florida. Fla. Entomol. 77**:** 460-474.

Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune. 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites. Appl. Environ. Microbiol. 69: 6018-6024.

Slaytor, M. 1992. Cellulose digestion in termites and cockroaches - what role do symbionts play? Comp. Biochem. Physiol. B 103**:** 775-784.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22**:** 4673-4680.

Tokura, M., M. Ohkuma, and T. Kudo. 2000. Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol. Ecol. 33: 233-240.

Vita

Marc Lewis Fisher

Marc Lewis Fisher was born to military parents on February 17, 1974, in Frankfurt, Germany. He spent most of his young life in Irving, Texas, where he graduated from Irving High School in 1992. After a two year stint serving an ecclesiastical mission for the Church of Jesus Christ of Latter-day Saints in Los Angeles, California, Marc completed his B.S. in Biology Composite Secondary Education at Brigham Young University in 1999. After teaching 7th and 8th grade science for one year at Colleyville Middle School, he continued his studies at Texas A&M University. He received his M.S. in Entomology in 2002 under the tutelage of Dr. Roger Gold. After working as an intern for Dow AgroSciences in Indianapolis during the summer of 2002, Marc started his Ph.D. studies at Virginia Tech as a GEM Fellow. His co-advisors were Drs. Dini Miller and Carlyle Brewster. He investigated the bacterial diversity in the gut of the eastern subterranean termite, *Reticulitermes flavipes*. During his Ph.D. studies, Marc was awarded the ESA 1st Place President's Prize twice, was a finalist for the Bayer Young Scientist of the Year Award, and won numerous scholarships, including the National Conference on Urban Entomology Ph.D. Scholarship, the Kosztarab Entomological Scholarship, the ESA Jeffery P. LaFage Scholarship, the Virginia Pest Management Association Scholarship, and the International Union for the Study of Social Insects Nutting Scholarship. Marc defended his dissertation on April 25, 2006.

Marc married Sarah Irene McBee on June 7, 1996 and is the father of five children, Avanlee, Dallin, Savannah, Christian, and Liriel.