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Genetic Variation and Species Distribution of Subterranean Termites in the Southeastern U.S. Focusing on Family Structure and the Invasive Formosan Subterranean Termite

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Entomology

by

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The Ohio State University
Bachelor of Science in Entomology, 2012

July 2015
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This thesis is approved for recommendation to the Graduate Council

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ABSTRACT

This work applies molecular genetic tools to distinguish the identity and understand the biology of termites, particularly *Reticulitermes* Holmgren and *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) in the southeastern U.S. Termites are important economic pests costing billions of dollars annually to Americans but also are important wood decomposers in natural settings. Molecular tools are essential for species identification because of the morphological ambiguities among species. The cryptic nest structure of subterranean termites which prevents adequate sampling makes molecular genetic tools essential to examine an entire colony.

A molecular diagnostic technique was created to differentiate *Coptotermes formosanus*, an invasive species in the U.S., from native subterranean termites. Using a multiplex PCR protocol, this method can distinguish *C. formosanus* even from a single specimen or sample lacking the diagnostic castes.

In southern Mississippi, a new termite species was observed and confirmed genetically. Using both morphological and molecular phylogenetic evidence, *Reticulitermes mississippiensis* Janowiecki, Szalanski, and Austin sp. nov. is described here as a new species.

The breeding structure of a termite colony refers to the number of male and female termites reproducing in the colony that contribute to the genetic diversity of the colony. While this is near impossible to determine from a field census, microsatellite DNA analysis has been previously applied to investigate this biological aspect in the North Carolina, South Carolina, Massachusetts, and Tennessee. Here, we apply these techniques to three species of *Reticulitermes* from three sites in northwest Arkansas. Generally, our results were similar to

those previously observed with 22% of the colonies being simple families (one male and one female), 72% extended families (the offspring of one male and one female reproducing) and 6% being mixed families (where multiple unrelated reproductives are reproducing in the colony). This study observed the first mixed family colonies of *Reticulitermes hageni* Banks. While these results show interesting trends of family structure for each species, more sampling is required to verify these observations.

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CHAPTER 1

INTRODUCTION

TERMITE BIOLOGY

Termites (Isoptera) are classified as social insects, meaning they share resources, cooperate in young rearing, have a division of labor, and overlapping generations (Suiter et al. 2002). The general caste system consists of workers, soldiers, and reproductives, each with their own tasks. The caste is determined during post-embryonic development (Laine and Wright 2003) by pheromones present in the colony (Suiter et al. 2002).

Termites feed on cellulose material, which makes them both important forest decomposers and pests of wooden structures. Lower termites (Cratomastotermitidae, Mastotermitidae, Termopsidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Archeorhinotermitidae, Stylotermitidae, Rhinotermitidae, and Serritermitidae) rely on anaerobic symbiotic protozoa in their hindgut to digest cellulose. Higher termites (Termitidae) lack these symbionts and digest cellulose with prokaryotic hindgut symbionts and endogenous cellulases (Bignell et al. 2010, Krishna et al. 2013).

In total, there are 3105 described species of termites grouped into 12 families, with the greatest abundance and diversity in tropical rainforests (Krishna et al. 2013). In the United States, there are approximately 50 species of native and introduced termites in three families (Termopsidae, Kalotermitidae, and Rhinotermitidae) of which only 18 species are structural pests (Thorne and Forschler 2001).

SUBTERRANEAN TERMITES

Subterranean termites, Rhinotermitidae Froggatt, are the most economically destructive family of termites and live in colonies in the soil. While these termites can be destructive to wooden structures, each individual is soft bodied and prone to desiccation. Subterranean termites build shelter tubes to prevent dessication as well as for protection (Suiter et al. 2002). Only rhinotermitids are native to Arkansas with the only known species being *Reticulitermes flavipes* (Kollar), *R. virginicus* (Banks), and *R. hageni* Banks.

RETICULITERMES IN THE UNITED STATES

Among U.S. Rhinotermitidae, *Reticulitermes* Holmgren is the most species-rich genus, currently represented by seven species: *R. flavipes*, *R. virginicus*, *R. hageni*, *R. malletei* (Howard and Clément), *R. nelsonae* Lim and Forschler, *R. hesperus* Banks, and *R. tibialis* Banks.

The eastern subterranean termite, *R. flavipes*, is the most widespread species found nearly ubiquitous in the U.S. (Banks and Snyder 1920, Krishna et al. 2013). As the primary structural pest, this species accounts for a majority of the termite damage in the U.S (Suiter et al. 2002). *Reticulitermes virginicus*, the dark southern subterranean termite, is found from Florida north to Ohio and west to Texas (Krishna et al. 2013). *Reticulitermes hageni*, the light southeastern subterranean termite, aptly named for its lighter colored alates as compared to *R. flavipes* and *R. virginicus*, has a range spanning the southeastern U.S. This species generally has smaller colonies and is uncommonly a structural pest, being confined primarily to forest environments (Scheffrahn and Su 1994). *Reticulitermes malletei* occurs along the Atlantic coast, known from Delaware, Georgia, Maryland, North Carolina, and South Carolina (Austin et al. 2007). This species was originally described from biochemical characters but was recently confirmed

genetically by Austin et al. (2007). *Reticulitermes nelsonae*, the most recently described species in the U.S., has only found in North Carolina, Georgia, and Florida. The western subterranean termite, *R. hesperus*, and the arid land subterranean termite, *R. tibialis*, are dominant in the western U.S. *Reticulitermes hesperus* is common along the Pacific coast, while *R. tibialis* is more common in the inter-mountain region in the western U.S. (Thorne and Forschler 2001). The western subterranean termite is the dominant pest species in western states and differs from many of the eastern species with a fall mating flight (Thorne and Forschler 2001).

ECONOMIC IMPORTANCE

Subterranean termites are economically important structural pests that cost Americans approximately $11 billion annually for prevention, treatment, and repair of termite damage (Su 2002). Although termites can be damaging urban pests, they have a crucial role in recycling nutrients by decomposing wood and other nutrients back to the soil (Thorne and Forschler 2001).

FORMOSAN SUBTERRANEAN TERMITE

The Formosan subterranean termite (FST), *Coptotermes formosanus* Shiraki, is an invasive subterranean termite that is originally from southern China and Taiwan. In the U.S. it is now found in North Carolina, South Carolina, Georgia, Florida, Alabama, Mississippi, Texas, Louisiana, and Hawaii after its introduction in the 1950s (Evans et al. 2013). The FST was also introduced into California, but was successfully eradicated (Atkinson et al. 1993, Haagsma et al. 1995).

Coptotermes formosanus is particularly successful because it produces much larger colonies than *Reticulitermes* species and forages a larger distance. The FST colonies can contain

several million termites whereas native *Reticulitermes* termite colonies usually only contain several hundred thousand individuals. They also can forage up to approximately 100 m, a larger range than native subterranean termites in the U.S. (Scheffrahn and Su 2013). The FST also has the ability to attack living trees (Lai et al. 1983) making it a pest of ornamental trees and pecan groves (Affeltranger et al. 1987). Economically, this species alone totals more than $2 billion in treatment and damage in the U.S. (Lax and Osbrink 2003, Raina 2004). Because of the economic importance of this invasive termite and the difficulty in differentiating it from native *Reticulitermes* termites, a molecular diagnostic tool is essential to monitor the spread of this species.

MOLECULAR GENETICS APPLIED TO TERMITE BIOLOGY

Traditional research methods have limitations when applied to termite investigations. Termites have a complex biology that is difficult to examine because their cryptic nest is nearly impossible to sample entirely (Thorne and Forschler 2001). Also, termite species are difficult to distinguish morphologically without the correct caste, which may not always be present in the nest. These biological complexities complicate termite research, leading to the need for novel techniques to fully study these colonies.

Molecular genetic techniques are an example of a novel and successful tool that have been used to make significant advances in understanding termite biology (Vargo and Husseneder 2009). The main contributions of molecular genetics include clarifying the systematics and taxonomy, understanding the process of caste differentiation, differentiating species, discovering relationships among population’s genetics, and unraveling the complicated family structure of

termites (Vargo and Husseneder 2009). Since the latter three topics are the focus of this thesis, they will be further investigated:

TERMITE IDENTIFICATION

Termite identification is difficult because there are few distinctive characters differentiating species. Keys (Scheffrahn and Su 1994) can effectively determine species of the soldier or alate caste, but these castes are not always present or abundant. Alates are ideal for identification, but are only present for a short time each year before and during the dispersal swarm. Analysis of DNA sequence variation is another method of determining species (Foster et al. 2004, King et al. 2007, Smith et al. 2010), but this method is time consuming and expensive for a large number of samples.

Molecular diagnostic techniques are a valuable tool for termite species identification. Previous methods have differentiated *Reticulitermes* spp. using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) (Szalanski et al. 2003) and the Formosan subterranean termite using two separate PCR reactions (Szalanski et al. 2004). Advantages of these methods include a lack of ambiguity, ability to determine species of any caste, and capability of identifying a single individual sample (Vargo and Husseneder 2009). Disadvantages include molecular methods being more expensive and limited by degradation of improperly preserved samples.

POPULATION GENETICS

Population genetics studies examine the relatedness, measured in similarity of genetic sequences, of an organism over a geographical range (Hoy 2013). For *Reticulitermes* in the U.S., studies have focused on *Reticulitermes* spp. over the entire country: *R. flavipes* (Austin 2005); *R. tibialis* (Austin et al. 2008). Studies have also focused on smaller ranges including the American

Great Plains (Austin et al. 2006b); Arkansas and Louisiana (Austin et al. 2004c, Szalanski et al. 2008a); Oklahoma (Austin et al. 2004b); Texas (Austin et al. 2004a); Missouri (Pinzon Florian 2007); Georgia (Lim 2011); Florida (Szalanski et al. 2008b); North Carolina (Vargo 2003b); California (Tripodi et al. 2006); Oregon and Washington (McKern et al. 2007); Wisconsin (Arango et al. 2015); and Indiana (Wang et al. 2009). *Reticulitermes* spp. population dynamics were also examined in the eastern Mediterranean and Middle East (Austin et al. 2006a). Thus far, population genetics was examined using scattered samples. A more robust, systematic sampling design could illustrate significant trends.

FAMILY STRUCTURE

Termite colonies are founded by a monogamous pair of alates. As a colony grows, secondary reproductives, termed neotenics, can either supplement or replace the primary reproducing king and queen in the colony (Hu and Forschler 2012). Initially, every colony begins as a simple family, with a single male and female reproducing. As the original pair is replaced or supplemented, the nest then contains an extended family, with nest mates no longer always being direct siblings. Colonies can also contain a mixed family when there are cohabiting individuals from unrelated reproductives (Vargo and Husseneder 2009). While the mechanism of mixing is still unclear, this may be caused by colony fusion (Matsuura and Nishida 2001, Deheer and Vargo 2004, Fisher et al. 2004).

The family structure of a colony is nearly impossible to determine without molecular genetic techniques due to the cryptic nature of a termite nest. However, the number of reproducing individuals in the colony can be determined using microsatellite DNA analysis. By examining the genotype of multiple individuals from the same colony at numerous microsatellite

loci with pedigree analysis, the reproductive relatedness, the degree of inbreeding, and the number of reproductives within a colony can be determined.

Colony structure has been examined in natural *Reticulitermes* populations in: North Carolina for undisturbed forest (N=98) (Vargo 2003b, Vargo and Carlson 2006) and urban sites (N=225) (Vargo 2003a, Parman and Vargo 2008); South Carolina (N=49) (Vargo et al. 2006); Nebraska (N=8) (Majid et al. 2013); as well as in laboratory colonies (N=13) (Long et al. 2006). Family structure has also been analyzed in *Coptotermes formosanus* in Japan (N=30) (Vargo et al. 2003a), Louisiana (N=25) (Vargo et al. 2003b, Husseneder et al. 2005), and Hawaii (N=20) (Broughton and Grace 1994, Vargo et al. 2003b).

From these previous studies, there are ~75% simple families, ~20% extended families, and ~2% mixed families (Vargo and Husseneder 2009). Additional research is needed to expand this analysis throughout the range of each respective termite species in order to understand this complex, cryptic aspect of termite biology.

RESEARCH OBJECTIVES

The objectives of this research are to:

1) develop a multiplex PCR protocol that could be used to identify the Formosan subterranean termite regardless of life stage in a single PCR reaction.
2) provide taxonomic description for *Reticulitermes mississippiensis* sp. nov., a new species of subterranean termite from southern Mississippi.
3) investigate the family structure of *Reticulitermes* spp. found in Arkansas.

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CHAPTER 2

MOLECULAR DIAGNOSTIC TECHNIQUE FOR THE DIFFERENTIATION OF THE FORMOSAN SUBTERRANEAN TERMITE, *COPTOTERMES FORMOSANUS* (ISOPTERA: RHINOTERMITIDAE) FROM OTHER SUBTERRANEAN TERMITES BY MULTIPLEX-PCR[1]

ABSTRACT

The Formosan subterranean termite *Coptotermes formosanus* Shiraki; (Isoptera: Rhinotermitidae), is a major pest that is spreading throughout the southeastern United States. Morphological identification of worker specimens is not possible using available taxonomic keys based on morphological traits. A multiplex PCR protocol was developed that can differentiate the Formosan subterranean termite from other termite species in a single PCR reaction. This multiplex PCR protocol simplifies previous molecular diagnostic techniques.

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[1]Janowiecki, M. A. and A. L. Szalanski. Published in Florida Entomologist 98(1): 387-388.

INTRODUCTION

The Formosan subterranean termite (FST), *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), is an invasive termite that was introduced into the continental United States in the 1950s (Evans et al. 2013). Since its introduction, it has spread to the southeastern US (including North Carolina, South Carolina, Georgia, Florida, Alabama, Mississippi, Louisiana, and Tennessee) and Texas (Evans et al. 2013). This termite is particularly damaging since it can also feed on the heartwood of living trees, rather than solely dead wood (Lai et al. 1983). Annually, Americans spend over $1 billion in preventing and treating this termite (Lax & Osbrink 2003). Furthermore, damage from this species cost Americans an additional $1 billion each year (Raina 2004).

Termite identification is difficult and various methods have previously been used to identify this pest species (Scheffrahn & Su 1994; Szalanski et al. 2003, 2004; Smith et al. 2010). Traditionally, taxonomic keys based on morphological traits are used to identify termite species (Scheffrahn & Su 1994), but these keys are developed for soldiers or alates, which are not always collected. Thus, small samples or samples lacking these castes are problematic. DNA sequence data have been used to identify FST (Smith et al. 2010). This process is time consuming and expensive requiring that every sample be sequenced. A molecular diagnostic technique to identify FST was developed by Szalanski et al. (2004), but still this method requires 2 polymerase chain reactions (PCRs), because the oligonucleotide primers annealing temperatures are incompatible. This duplication made the process more time consuming and increased the chance for mistakes.

The objective of this study was to develop a multiplex PCR protocol that could be used to identify FST regardless of life stage. The technique, requiring only a single PCR reaction, is simpler than previous molecular methods, and will facilitate monitoring of this invasive termite.

MATERIALS AND METHODS

Termites were collected from locations in North America, South America, Africa, Asia, Australia, and the Middle East (Table 1). Identification was conducted using the keys of Scheffrahn & Su (1994). Voucher specimens are housed in the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, Arkansas, U.S.

Samples preserved in ethanol were dried on filter paper. DNA was extracted using DNeasy® (Qiagen Sciences, Germantown, Maryland), resuspended in 10mM Tris-HCL (pH 8.0), and stored at -20 °C. Universal termite oligonucleotide primers were designed using composite termite sequences in Geneious (v6.1.7, Invitrogen Corp., Grand Island, New York): 16S 104F (5'-CCTCYCATCRCCCCAACRAA-3') and 16S 368R (5'-TTGAAGGGCCGCGGTATYTT-3'). A 16S FST specific primer was also used: FST-F (5'-TAAAACAAACAAACAACAAACAAAC-3') (Szalanski et al. 2004). Polymerase chain reaction was performed at 94 °C for 2 min; followed by 40 cycles at 94 °C for 45 s, 50 °C for 45 s, and 72°C for 60 s. The final extension at 72 °C f was for 5 min.

The new method was validated by screening a broad geographical sampling of FST and *Reticulitermes* species from the US. Samples were visualized on a 2% agarose gel with ethidium bromide staining (Fig. 1). The FST samples yielded 2 amplicons of 262 and 221 bp in size. Other Nearctic termite species north of Mexico [*Coptotermes gestroi* Wasmann, *Reticulitermes flavipes* (Kollar), *R. virginicus* Banks, *R. tibialis* Banks, *R. hageni* Banks, *Heterotermes aureus* (Snyder),

R. hesperus Banks, and *R. malletei* Howard and Clement] were used (Table 1) and generated only a single amplicon of 262 bp. Additional *Coptotermes* spp. [*C. michaelseni* Silvestri, *C. lacteus* (Froggatt), *C. testaceus* (L.), *C. intermedius* Silvestri, *C. heimi* (Wasmann)] produced only the single universal amplicon of 262 bp. The negative control did not produce a detectable amplicon, indicating no contamination. A total of 1,373 16S sequences from 9 *Coptotermes*, 6 *Reticulitermes*, and 4 *Heterotermes* species from GenBank and from our DNA sequence database (ALS unpublished) (Table 1) were analyzed using Geneious software to confirm that the FST oligonucleotide primers would be specific for the *C. formosanus* (FST) sequences and not the other taxa for PCR amplification.

RESULTS AND DISCUSSION

The results show that the universal primers produced a 262 bp band in all species tested (Table 1) whereas the FST specific primer produces an additional band (221 bp) only in FST (Fig. 1), indicating that this primer combination and PCR reaction successfully distinguish FST from other termites in this study.

This new molecular method simplifies previous methods of identification (Szalanski et al. 2004; Evans et al. 2013), in that it can be completed in a single PCR reaction and allows identification of worker specimens that cannot be keyed morphologically to species. Proper identification that is simple and economical can be useful for monitoring the spread of this invasive to new areas.

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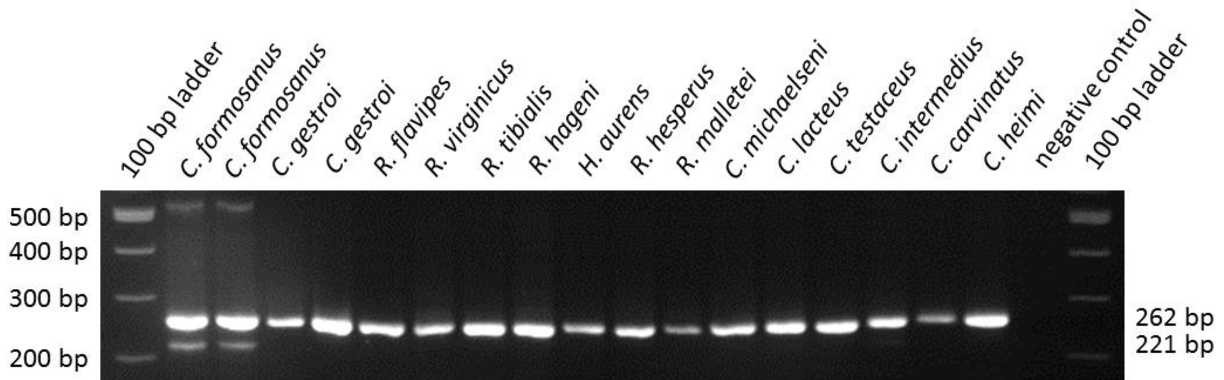
TABLE 2.1. *Coptotermes*, *Reticulitermes*, and *Heterotermes* samples subjected to DNA sequencing and PCR analysis for Formosan subterranean termite specific PCR diagnostic analysis.

Species	Number PCR Screened	Number Sequence Screened
C. formosanus	9	57
C. gestroi	2	19
C. michaelseni	1	2
C. lacteus	1	6
C. testaceus	1	29
C. intermedius	1	1
C. curvignathus	1	2
C. heimi	1	2
R. flavipes	16	747
R. virginicus	15	108
R. tibialis	12	95
R. hageni	4	82
R. hesperus	1	75
R. malletei	4	31
H. aureus	1	1
H. tenius	0	10
H. cardini	0	18
H. convexinotatus	0	21

FIGURE 2.1. Ethidium bromide-stained agarose gel (2%) illustrating a common amplicon of 262 bp from the mtDNA 16S gene for various termite species and unique amplicon of 221 bp specific for the Formosan subterranean termite.


100 bp ladder
C. formosanus
C. formosanus
C. gestroi
C. gestroi
R. flavipes
R. virginicus
R. tibialis
R. hageni
H. aurens
R. hesperus
R. malletei
C. michaelseni
C. lacteus
C. testaceus
C. intermedius
C. carvinatus
C. heimi
negative control
100 bp ladder
500 bp
400 bp
300 bp
200 bp
262 bp
221 bp

CHAPTER 3

A NEW SPECIES OF *RETICULITERMES* (ISOPTERA: RHINOTERMITIDAE) FROM SOUTHERN MISSISSIPPI [1]

ABSTRACT

Reticulitermes mississippiensis sp. nov. is a new species described from specimens collected in southern Mississippi. Descriptive castes (soldiers and alates) are described and molecular phylogenetic evidence is provided to establish this as a new species of Rhinotermitidae (Isoptera).

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[1]Janowiecki, M. A. J. W. Austin, and A. L. Szalanski. To be submitted to Florida Entomologist.

INTRODUCTION

Termites are social insects that feed primarily on cellulose material (Suiter et al. 2002). Subterranean termites (Rhinotermitidae) include the most destructive species of termites and nest in contact with soil (Thorne and Forschler 2001). Subterranean termites cost approximately $11 billion annually in the United States for prevention, treatment, and repair of termite damage (Su 2002). *Reticulitermes* Holmgren is the most species-rich genus of subterranean termite in the U.S. currently represented by seven species: *R. flavipes* (Kollar 1837), *R. virginicus* (Banks 1907), *R. hageni* Banks 1920, *R. hesperus* Banks 1920, *R. tibialis* Banks 1920, *R. malletei* (Howard and Clément 1985), and *R. nelsonae* (Lim and Forschler 2012). *Reticulitermes arenincola* Goellner was considered to be another U.S. species of *Reticulitermes* but is currently generally accepted as a *nomen dubium* (“doubtful name”) (Austin et al. 2007, Vargo and Husseneder 2009).

The history and taxonomy of *Reticulitermes* has most recently been published by Lim (2011). Five of the seven U.S. species were described in the nineteenth or early twentieth century using only morphological characters. The remaining species were described recently using a combination of novel techniques.

Reticulitermes malletei was described in 1985 by Howard and Clément using biochemical characters including cuticular hydrocarbons, soldier defensive substance, and sexual pheromone; and behavioral characters including worker aggression and swarming period (Clément et al. 1986). Although this species was described in 1985, it was not generally accepted as a species by termite experts until it was validated by Austin et al. (2007). This later study also used cuticular hydrocarbons and combined morphological characters with mtDNA to justify *R. malletei* as a valid species.

Most recently, *Reticulitermes nelsonae* was described by Lim and Forschler (2012). This species, isolated to North Carolina, Georgia, and Florida, was described using morphological and behavioral characters as well as mtDNA.

J. L. Ethridge, at the Costal Research and Extension Center, Mississippi State University, Poplarville, MS, sent several samples of *Reticulitermes* termites to the Insect Genetics Laboratory, University of Arkansas, for species identification. From those samples collected by J. L. Ethridge, and additional samples, we provide a formal description for *Reticulitermes mississippiensis* Janowiecki, Szalanski, and Austin sp. nov., a proposed new species collected in Mississippi using both morphological and genetic characters.

MATERIALS AND METHODS

SPECIMENS

Fourteen samples of *R. mississippiensis* sp. nov. were collected from three locations in Stone and Hancock counties in southern Mississippi and preserved in ethanol (see Table 3.1). Morphological data for this study was obtained from 24 soldiers and 20 alates. Remaining castes were used for DNA analysis. Voucher specimens are housed in the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, Arkansas, U.S. Samples will also be deposited in the American Museum of Natural History (AMNH), New York, New York, U.S. and the National Museum of Natural History (NMNH), Smithsonian Institution, Washington, DC, U.S.

MORPHOMETRICS

Specimens were examined morphologically with a binocular dissecting microscope (SM-1TZ, AmScope, Irvine, CA, U.S.) equipped with a camera (MU1000, 10MP, AmScope, Irvine,

CA, U.S.) using ToupView v. 3.2.1476 (AmScope, Irvine, CA, U.S.). Soldier and alate measurements were based on Lim (2011) and included: soldier head capsule length (sl), soldier head capsule width (sw), alate body length (abl), alate body length including wings (ablw), alate forewing length (afw), alate hindwing length (ahw).

BEHAVIOR

Reticulitermes mississippiensis sp. nov. alates that had not yet swarmed were collected from colonies at the Hancock County Mississippi State University (MSU) Extension Center.

DICHOTOMOUS KEY

Because of the overlapping ranges of morphological characters, no dichotomous key is provided to differentiate *R. mississippiensis* sp. nov. from other *Reticulitermes* spp. Rather, genetic identification is ideal for these morphologically ambiguous species.

MOLECULAR DATA

Genomic DNA was extracted from a head from an individual specimen using a salting-out procedure with in-house reagents (Sambrook et al. 2001). Two regions of mtDNA were amplified using the primers 16S: LR-J-13007 (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati and Smith 1995) and LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3') (Simon et al. 1994); and COII mtDNA: TL2-J-3037 (5'-ATGGCAGATTAGTGCAATGG-3') and TK-N-3785 (5'-GTTTAAGAGACCAGTACTTG-3') (Lim and Forschler 2012). Consensus sequences were obtained from an alignment of sequences in both directions for each sample using Geneious v6.1.8 (Kearse et al. 2012). Sequences were subjected to a BLAST search of DNA sequences deposited to GenBank to determine their similarity.

For the molecular phylogenetic analysis, our sequences and the outgroup taxa *Coptotermes formosanus* (FJ870592) and *Heterotermes tenuior* (AB050714) were used.

Additional *Reticulitermes* sequences were obtained from GenBank (Table 3.2). Multiple alignment of sequences was done using Geneious v6.1.8 (Kearse et al. 2012) with a cost matrix of 6% similarity, a gap open penalty of 12, and a gap extension penalty of three. Maximum parsimony analysis was conducted using PAUP v4b10 (Swofford 2001). The reliability of trees was tested using bootstrap analysis (Felsenstein 1985). Parsimony bootstrap analysis included 1,000 resamplings using the Branch and Bound algorithm of PAUP. Bayesian phylogenetic analysis was conducted with the MrBayes (Ronquist and Huelsenbeck 2003) plug-in within Geneious with 100,000 burn-in and 1,000,000-replications using a GTR+G model based on AIC results from jModelTest v2.1.3 (Darriba et al. 2012). A neighbor joining tree was also constructed with Geneious using 1000 bootstrap replicates.

RESULTS

MORPHOMETRICS

Reticulitermes mississippiensis sp. nov. measurements were similar to other species of *Reticulitermes* in the southeastern U.S. (Table 3.3). Both measures of soldier head capsule (sl and sw) were most similar to *R. malletei*, whereas alate characteristics were similar to *R. flavipes*, *R. hageni*, *R. malletei*, and *R. hageni* respectively for abl, ablw, afw, and ahw.

The soldier (Figure 3.1) head measured 1.30-1.62 mm for sl and 0.82-0.97 mm for sw with averages of 1.49 mm and 0.90 mm, respectively. The alate (Figure 3.3) body length measured 4.03-6.36 mm without wings (abl) and 7.26-8.61 mm including wings (ablw) with averages of 4.59 mm and 7.97 mm, respectively. The alate forewing was longer, measuring 5.81-6.98 mm (average: 6.47mm), than the hind wing, measuring 5.07-6.48 mm (average: 5.95 mm) (Table 3.3).

BEHAVIOR

Sclerotized alates that had not yet swarmed were collected in two colonies in Hancock CO, MS, U.S. on 27 April 2004. This implies that these colonies were near swarming, likely within 30 days (Lim 2011), estimating the flight window as late April to early May.

MOLECULAR DATA

The 14 samples of *R. mississippiensis* sp. nov. consisted of three unique 16S haplotypes with a total of eight polymorphic base pairs. For all trees, *R. mississippiensis* sp. nov. forms a distinct clade from all other southeastern *Reticulitermes* (Figure 3.3-3.5). The Bayesian phylogenetic tree (Figure 3.3) shows *R. mississippiensis* sp. nov. in a distinct clade with *R. nelsonae* as a sister taxon. One *R. nelsonae* (JF796236) groups intermediately between the two species, and requires future analysis to confirm that this is not a sequencing error. The maximum parsimony tree (Figure 3.4) also shows *R. nelsonae* as a sister taxon, but with *R. malletei* as the sister taxon to both *R. mississippiensis* sp. nov. and *R. nelsonae*. This aligns with the soldier morphologically characters grouping *R. mississippiensis* sp. nov. with *R. malletei*. The neighbor-joining phylogenetic tree (Figure 3.5) groups things as the maximum parsimony tree does with *R. nelsonae* as a sister taxon and *R. malletei* as a sister taxon to both species.

SYSTEMATICS

Reticulitermes mississippiensis Janowiecki, Szalanski, and Austin sp. nov.

Figures 3.1-3.2

Soldier (Table 3.3, Figure 3.1): The soldier head capsule length (sl) and width (sw) is approximately the same as *R. malletei* at an average of 1.495 mm 0.899 mm respectively.

Alate (Table 3.3, Figure 3.2): *R. mississippiensis* has a dark brown body with 14 antennal segments. Flights occur in late April to May, overlapping with other species of *Reticulitermes*. Alate body length (abl) is typically 0.2 mm less than *R. flavipes* and 0.6 mm greater than *R. virginicus*, *R. hageni, R. malleti*, and *R. nelsonae*. The remaining *R. mississippiensis* sp. nov. alate measurements (ablw, afw, ahw) overlap with the remaining species more closely and resemble both *R. hageni* and *R. malletei* (Table 3.3).

DISTRIBUTION

This species has only been found in Stone and Hancock counties in southern Mississippi, U.S. despite extensive sampling in the southeastern U.S. (A. L. Szalanski, Professor of Entomology, University of Arkansas, U.S., personal communication) and in southern Mississippi (Howard et al. 1982, Wang and Powell 2001, Wang et al. 2003).

TYPES

The holotype will be deposited in the NMNH and paratypes will be deposited in both the UARK Arthropod Museum and the AMNH.

GENETICS

Figures 3.3 and 3.4 show phylogenetic trees for *Reticulitermes* for 16S and COII mtDNA. In both trees, *R. mississippiensis* sp. nov. forms a distinct clade from all other species, showing genetic isolation of this species.

DISCUSSION

From the comparison of southeastern species of *Reticulitermes*, it is clear that these species are difficult to differentiate morphologically. When only using morphological characters, Lim (2011) found that it required 6-29 specimens (depending on species) to identify with 95%

confidence. This necessitates novel techniques for identification of these important pest species, this study being an application of genetic methods. Morphologically and genetically, *R. mississippiensis* sp. nov. is a distinct species from all other southeastern *Reticulitermes*.

R. mississippiensis sp. nov. differs from the other southeastern U.S. species of *Reticulitermes* in its restricted range. Despite extensive sampling, this species has only been found in two counties in southern Mississippi. Wang et al. (2003) collected 103 samples from the two counties *R. mississippiensis* sp. nov. occurs and an additional 155 samples from two adjacent counties and did not report finding any species besides *R. flavipes, R. hageni,* and *R. virginicus.* Additional studies (Howard et al. 1982, Wang and Powell 2001) examined *Reticulitermes* in southern Mississippi and also found only these three species. The limited range of *R. mississippiensis* sp. nov. is not an artifact of a lack of sampling, but rather likely the entire range of the species. This aspect contrasts from all other species, which have multi-state ranges with *R. nelsonae* being the next restricted, only known to occur in three states. While the mechanism causing this isolation is unknown, this introduces the notion that more "hidden" species occur undetected in under sampled areas.

Species identification is a critical component of effective termite control. While *R. flavipes* is the most common home invader, the other *Reticulitermes* species are also known to attack wooden structures (Suiter et al. 2002). Proper description and identification of species is an important first step in the control of this economically important pest.

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TABLE 3.1. Specimen data for collections of *Reticulitermes mississippiensis* sp. nov. in Mississippi.

Location	County	Sampling events	Alates	Soldiers	Neotenics	Workers	Larvae	Total
Stennis Space Center	Hancock	8	0	11	14	438	75	538
MSU County Extension Office	Hancock	5	247	18	107	319	62	753
Flint Creek Park, Wiggins	Stone	1	0	1	0	16	0	17
Totals		14	247	30	121	773	137	1308

TABLE 3.2. Sequence names, identification, locality, and GenBank accession number for COII mtDNA sequences used for the phylogenetic trees in this study.

No	Sequence names	Species	GenBank acc no.*	Source**
1	*R. mississippiensis* 1356	*R. mississippiensis* sp. nov.	TBD	this paper
2	*R. mississippiensis* 904	*R. mississippiensis* sp. nov.	TBD	this paper
3	*R. mississippiensis* 1061	*R. mississippiensis* sp. nov.	TBD	this paper
4	*R. mississippiensis* 1052	*R. mississippiensis* sp. nov.	TBD	this paper
5	*R. mississippiensis* 905	*R. mississippiensis* sp. nov.	TBD	this paper
6	*R. mississippiensis* 1351	*R. mississippiensis* sp. nov.	TBD	this paper
7	*R. mississippiensis* 1350	*R. mississippiensis* sp. nov.	TBD	this paper
8	*R. mississippiensis* 1168	*R. mississippiensis* sp. nov.	TBD	this paper
9	*R. mississippiensis* 1348	*R. mississippiensis* sp. nov.	TBD	this paper
10	*R. mississippiensis* 1043	*R. mississippiensis* sp. nov.	TBD	this paper
11	*R. mississippiensis* 910	*R. mississippiensis* sp. nov.	TBD	this paper
12	*R. nelsonae* JF796236	*R. nelsonae*	JF796236	1
13	*R. nelsonae* JF796235	*R. nelsonae*	JF796235	1
14	*R. nelsonae* JF796229	*R. nelsonae*	JF796229	1
15	*R. nelsonae* JF796233	*R. nelsonae*	JF796233	1
16	*R. nelsonae* JF796232	*R. nelsonae*	JF796232	1
17	*R. nelsonae* JF796231	*R. nelsonae*	JF796231	1
18	*R. nelsonae* JF796230	*R. nelsonae*	JF796230	1
19	*R. malletei* JF796228	*R. malletei*	JF796228	1
20	*R. malletei* 4899	*R. malletei*	N.A.	unpublished
21	*R. malletei* 4900	*R. malletei*	N.A.	unpublished
22	*R. malletei* FJ606690	*R. malletei*	FJ606690	unpublished
23	*R. malletei* 1273b	*R. malletei*	N.A.	unpublished
24	*R. malletei* 4901	*R. malletei*	N.A.	unpublished
25	*R. malletei* JF796227	*R. malletei*	JF796227	1
26	*R. malletei* GU550074	*R. malletei*	GU550074	2

TABLE 3.2. (Cont.)

No	Sequence names	Species	GenBank acc no.*	Source**
27	*R. malletei* JF796226	*R. malletei*	JF796226	1
28	*R. hageni* EU689026	*R. hageni*	EU689026	2
29	*R. hageni* JF796225	*R. hageni*	JF796225	1
30	*R. hageni* JF796224	*R. hageni*	JF796224	1
31	*R. virginicus* JF796223	*R. virginicus*	JF796223	1
32	*R. virginicus* JF796222	*R. virginicus*	JF796222	1
33	*R. flavipes* JQ280746	*R. flavipes*	JQ280746	3
34	*R. flavipes* JQ280744	*R. flavipes*	JQ280744	3
35	*R. flavipes* AF107479	*R. flavipes*	AF107479	4
36	*R. tibialis* DQ493741	*R. tibialis*	DQ493741	unpublished
37	*R. tibialis* HM208248	*R. tibialis*	HM208248	unpublished
38	*Heterotermes tenuior* AB050714	*H. tenuior*	AB050714	unpublished
39	*Coptotermes formosanus* FJ870592	*C. formosanus*	FJ870592	5

*TBD indicates will be determined when submitted to GenBank
N.A. indicates samples has no GenBank accession number

**1(Lim and Forschler 2012)
2(Sillam-Dussès and Forschler 2010)
3(Perdereau et al. 2013)
4(Jenkins et al. 1999)
5(Zhu et al. 2010)

TABLE 3.3. Results of measurements of soldiers (n=24) and alates (n=20) comparing *R. mississippiensis* sp. nov. to other species of *Reticulitermes* from the southeastern U.S. (* = from Lim 2011). **Bold** indicates most similar.

Species	sl (mm) (mean ± sd)	sw (mm) (mean ± sd)	abl (mm) (mean ± sd)	ablw (mm) (mean ± sd)	afw (mm) (mean ± sd)	ahw (mm) (mean ± sd)
R. mississippiensis sp. nov.	**1.495 ± 0.076**	**0.899 ± 0.038**	**4.594 ± 0.475**	**7.967 ± 0.345**	**6.469 ± 0.342**	**5.953 ± 0.353**
*R. flavipes**	1.693 ± 0.119	1.044 ± 0.074	**4.783 ± 0.383**	8.973 ± 0.402	6.810 ± 0.331	6.550 ± 0.332
*R. virginicus**	1.625 ± 0.068	0.920 ± 0.039	4.021 ± 0.214	7.414 ± 0.213	5.532 ± 0.193	5.418 ± 0.193
*R. hageni**	1.434 ± 0.092	0.862 ± 0.030	4.083 ± 0.323	**7.810 ± 0.318**	5.965 ± 0.263	**5.739 ± 0.257**
*R. malletei**	**1.490 ± 0.058**	**0.879 ± 0.029**	4.023 ± 0.302	8.238 ± 0.394	**6.375 ± 0.328**	6.100 ± 0.339
*R. nelsonae**	1.407 ± 0.127	0.784 ± 0.054	3.928 ± 0.236	7.080 ± 0.291	5.430 ± 0.212	5.315 ± 0.297

sl = soldier head capsule length
sw = soldier head capsule width
abl = alate body length
ablw = alate body length including wings
afw = alate forewing length
ahw = alate hindwing length

FIGURE 3.1. Soldier of *R. mississippiensis* sp. nov. showing lateral view (A) and head capsule (B). Photographs by James Austin, used with permission.

A

B

FIGURE 3.2. Alate of *R. mississippiensis* sp. nov. showing dorsal view (A), lateral view (B), forewing (C), and hind wing (D). Photographs by James Austin, used with permission.

A

B

C

D

FIGURE 3.3. Bayesian phylogenetic tree of *Reticulitermes* inferred from COII mtDNA. Posterior bootstrap values are provided.

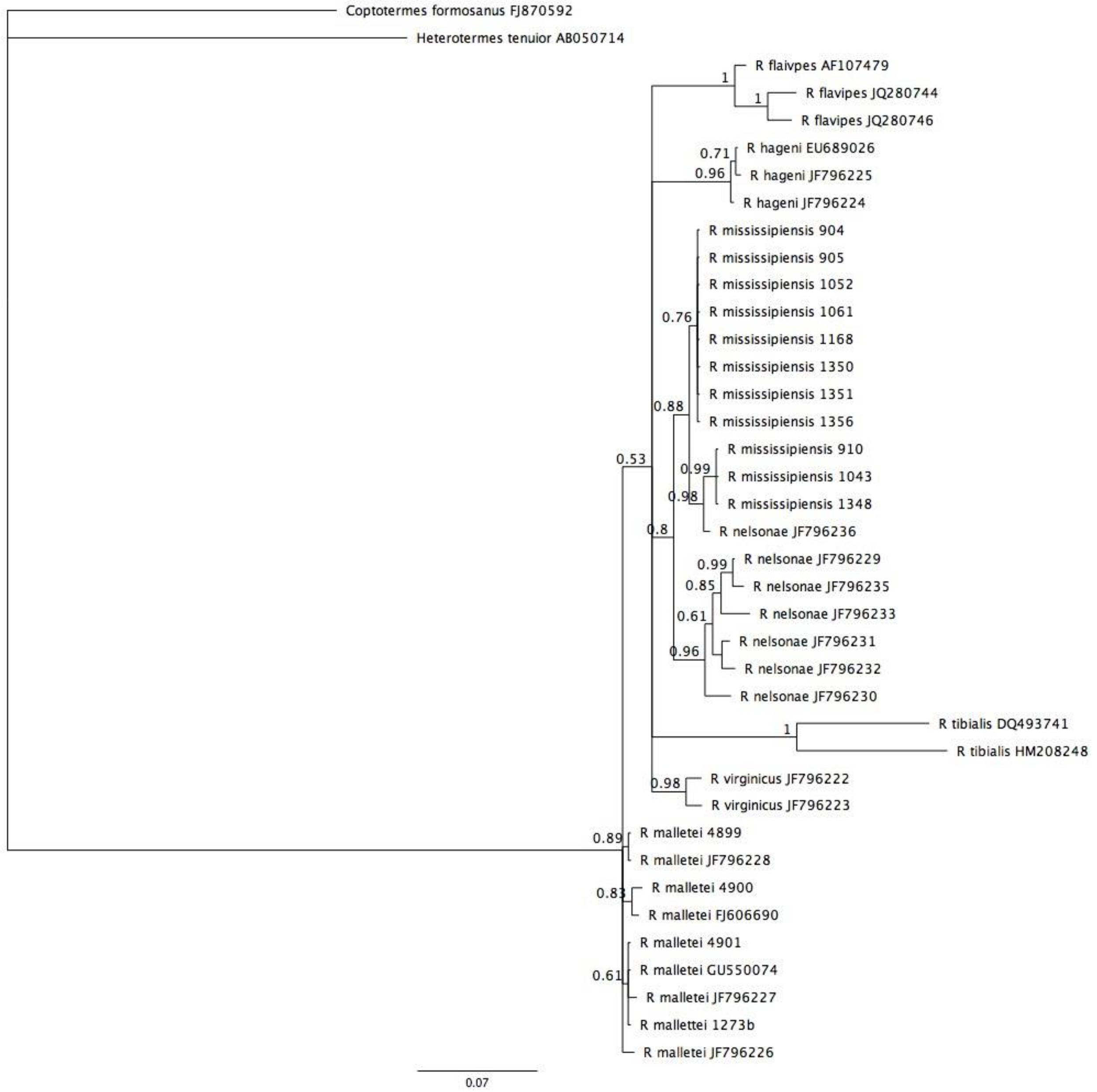

Coptotermes formosanus FJ870592
Heterotermes tenuior AB050714
1
R flaivpes AF107479
1
R flavipes JQ280744
R flavipes JQ280746
0.71
R hageni EU689026
0.96
R hageni JF796225
R hageni JF796224
R mississipiensis 904
R mississipiensis 905
R mississipiensis 1052
0.76
R mississipiensis 1061
R mississipiensis 1168
R mississipiensis 1350
R mississipiensis 1351
0.88
R mississipiensis 1356
0.53
R mississipiensis 910
0.99
R mississipiensis 1043
0.98
R mississipiensis 1348
0.8
R nelsonae JF796236
0.99
R nelsonae JF796229
0.85
R nelsonae JF796235
R nelsonae JF796233
0.61
R nelsonae JF796231
0.96
R nelsonae JF796232
R nelsonae JF796230
1
R tibialis DQ493741
R tibialis HM208248
0.98
R virginicus JF796222
R virginicus JF796223
0.89
R malletei 4899
R malletei JF796228
0.83
R malletei 4900
R malletei FJ606690
R malletei 4901
0.61
R malletei GU550074
R malletei JF796227
R mallettei 1273b
R malletei JF796226
0.07

FIGURE 3.4. Maximum parsimony phylogenetic tree of *Reticulitermes* inferred from COII mtDNA. Bootstrap values are provided.

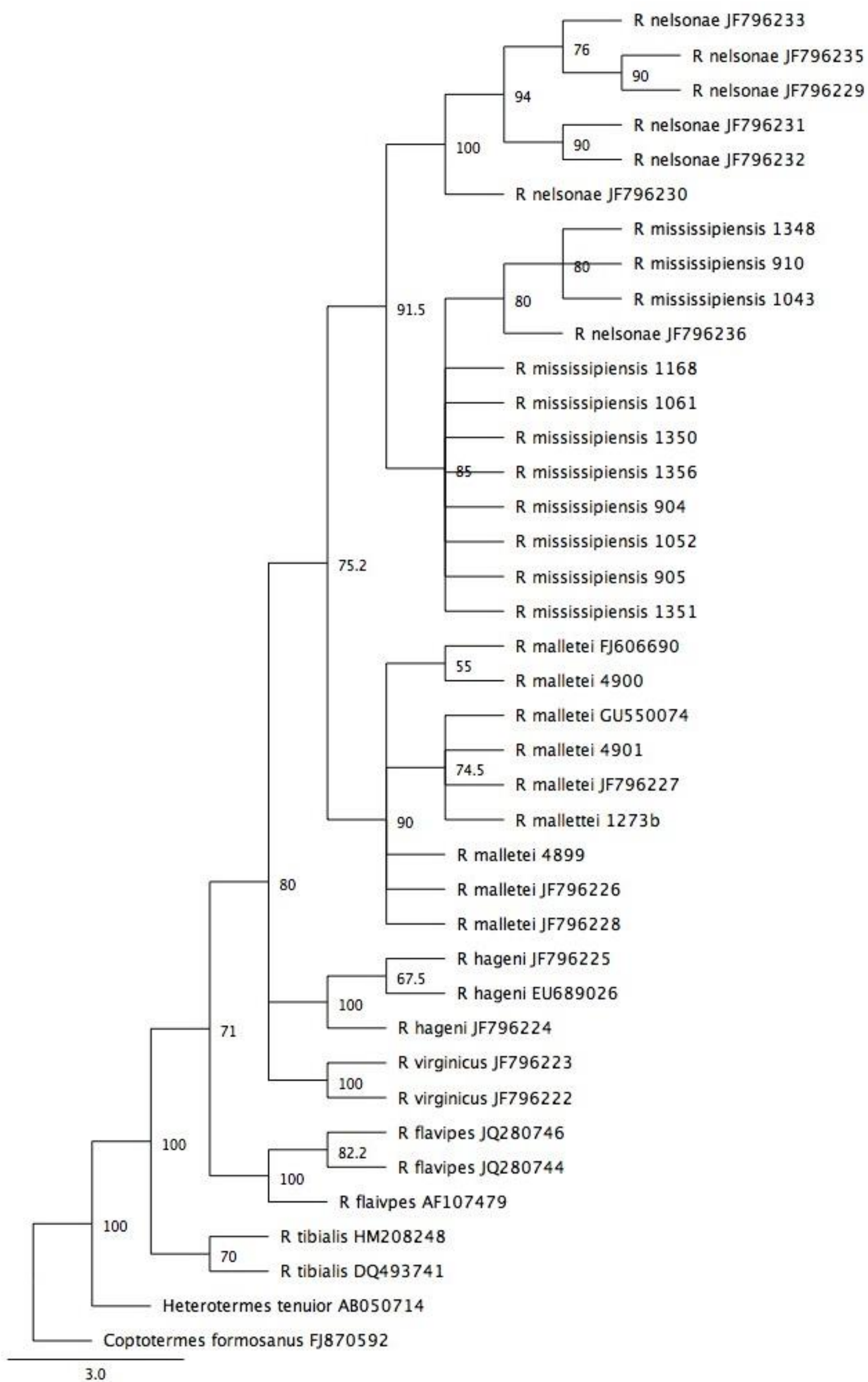
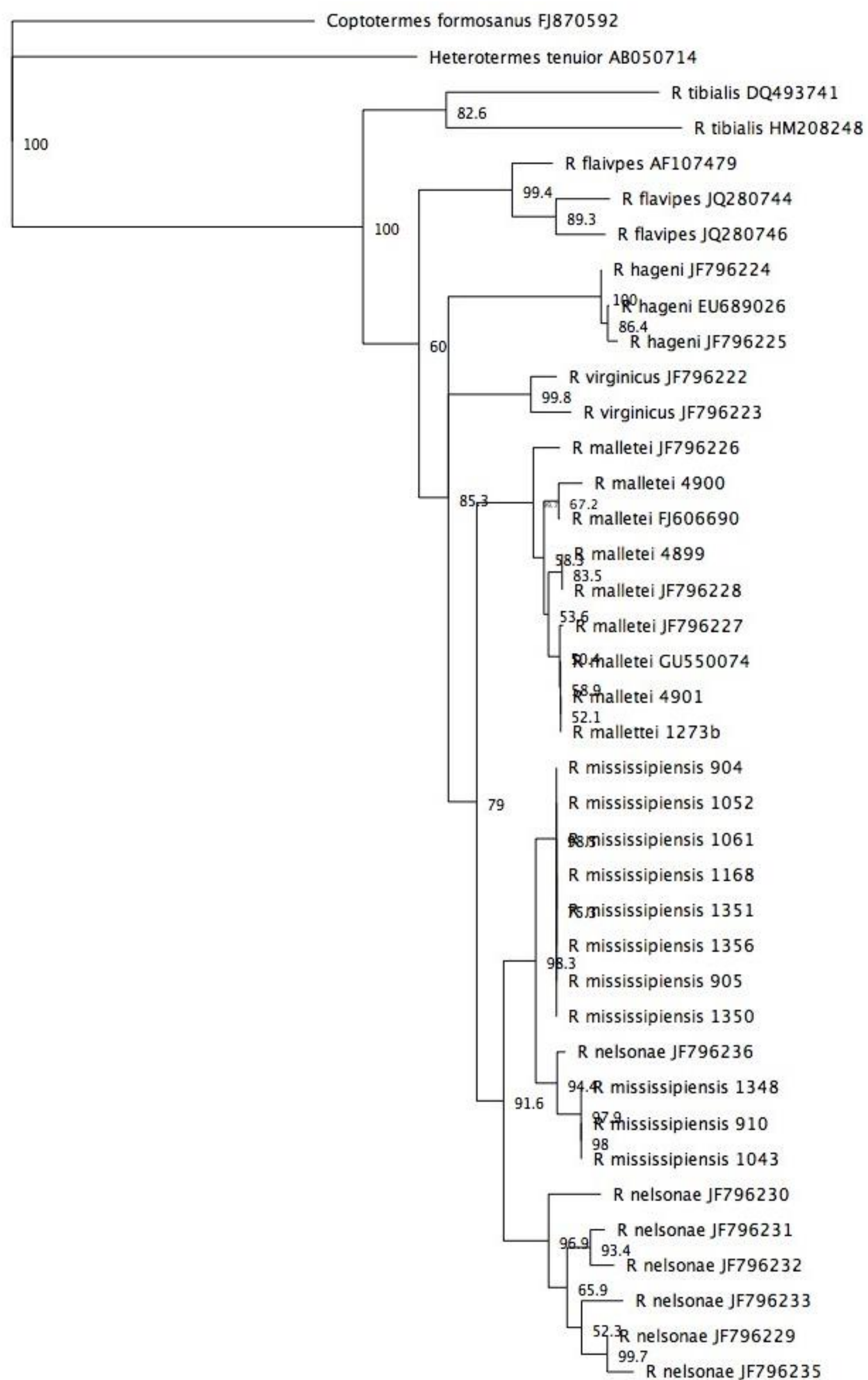

R nelsonae JF796233
76
R nelsonae JF796235
90
R nelsonae JF796229
94
R nelsonae JF796231
90
R nelsonae JF796232
100
R nelsonae JF796230
R mississipiensis 1348
80
R mississipiensis 910
80
R mississipiensis 1043
91.5
R nelsonae JF796236
R mississipiensis 1168
R mississipiensis 1061
R mississipiensis 1350
85
R mississipiensis 1356
R mississipiensis 904
R mississipiensis 1052
75.2
R mississipiensis 905
R mississipiensis 1351
R malletei FJ606690
55
R malletei 4900
R malletei GU550074
R malletei 4901
74.5
R malletei JF796227
R mallettei 1273b
90
R malletei 4899
80
R malletei JF796226
R malletei JF796228
R hageni JF796225
67.5
R hageni EU689026
100
R hageni JF796224
71
R virginicus JF796223
100
R virginicus JF796222
R flavipes JQ280746
82.2
100
R flavipes JQ280744
100
R flaivpes AF107479
100
R tibialis HM208248
70
R tibialis DQ493741
Heterotermes tenuior AB050714
Coptotermes formosanus FJ870592
3.0

FIGURE 3.5. Neighbor-joining phylogenetic tree of *Reticulitermes* inferred from COII mtDNA. Bootstrap values are provided.


Coptotermes formosanus FJ870592
Heterotermes tenuior AB050714
R tibialis DQ493741
R tibialis HM208248
R flaivpes AF107479
R flavipes JQ280744
R flavipes JQ280746
R hageni JF796224
R hageni EU689026
R hageni JF796225
R virginicus JF796222
R virginicus JF796223
R malletei JF796226
R malletei 4900
R malletei FJ606690
R malletei 4899
R malletei JF796228
R malletei JF796227
R malletei GU550074
R malletei 4901
R mallettei 1273b
R mississipiensis 904
R mississipiensis 1052
R mississipiensis 1061
R mississipiensis 1168
R mississipiensis 1351
R mississipiensis 1356
R mississipiensis 905
R mississipiensis 1350
R nelsonae JF796236
R mississipiensis 1348
R mississipiensis 910
R mississipiensis 1043
R nelsonae JF796230
R nelsonae JF796231
R nelsonae JF796232
R nelsonae JF796233
R nelsonae JF796229
R nelsonae JF796235
100
82.6
100
99.4
89.3
86.4
60
99.8
85.3
67.2
83.5
53.6
52.1
79
91.6
94.4
98
96.9
93.4
65.9
99.7
0.03

CHAPTER 4

COLONY STRUCTURE OF *RETICULITERMES* (ISOPTERA: RHINOTERMITIDAE) IN NORTHWEST ARKANSAS[1]

ABSTRACT

Termites, as social insects, have a complicated life cycle that is difficult to study with traditional research methods. A termite colony can consist of a simple family (one male and one female), an extended family (multiple males and/or multiple females) or a mixed family (unrelated reproductives). While this is near impossible to determine from collecting and censusing colonies in the field, microsatellite DNA genotyping methods have been previously developed and applied to termites along the east coast. In this study, we apply these methods to three species of *Reticulitermes* from three forested sites in northwest Arkansas. In our limited sampling, we found 22% of *Reticulitermes* in northwest Arkansas were simple families, 72% were mixed families and 6% were mixed families. This study observed the first *R. hageni* mixed family as well as the first observation of a single termite colony containing multiple mtDNA haplotypes. Further sampling is required to strengthen these observations into general trends for family structure of *Reticulitermes* in northwest Arkansas.

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[1]Janowiecki, M. A., A. D. Tripodi, and A. L. Szalanski. To be submitted to the Journal of the Kansas Entomological Society.

INTRODUCTION

Termites, as social insects, have a complicated life cycle that is difficult to study with traditional research methods. Initially, a termite colony is founded by a monogamous pair of winged alates, forming a simple family (Suiter et al. 2002). This pair can be replaced by secondary reproductives, changing the breeding structure of the colony to include an extended family (Vargo and Husseneder 2009). Less commonly, colonies can consist of cohabiting individuals from unrelated reproductives. These are referred to as mixed families, likely caused by colony fusion (Matsuura and Nishida 2001, Deheer and Vargo 2004, Fisher et al. 2004).

While this structure is nearly impossible to determine from collecting and censusing colonies in the field, microsatellite DNA genotyping methods have been developed and applied to termites along the east coast (Vargo and Husseneder 2009). Microsatellite loci were initially developed for *R. flavipes* and *R. santonensis* (Vargo 2000, Dronnet et al. 2004) and have been extended to other species of *Reticulitermes*.

Family structure has been examined in natural *Reticulitermes* populations in: North Carolina for undisturbed forest (N=98) (Vargo 2003b, Vargo and Carlson 2006) and urban sites (N=225) (Vargo 2003a, Parman and Vargo 2008); South Carolina (N=49) (Vargo et al. 2006); Nebraska (N=8) (Majid et al. 2013); and in laboratory colonies (N=13) (Long et al. 2006). From these previous studies, there are approximately 75% simple families, 20% extended families, and 2% mixed families (Vargo and Husseneder 2009). Additional research is needed to expand this analysis throughout the range of each respective termite species in order to understand the complex, cryptic nature of termite biology.

In this study, we apply these methods to three species of *Reticulitermes* from three forested sites in northwest Arkansas.

MATERIALS AND METHODS

SAMPLES

Termites were collected from three sites in northwest Arkansas (Figure 4.1): Lake Wedington unit of the Ozark St. Francis National Forest (OSFNF), Lee Creek area of OSFNF, and the Arkansas Agricultural Research and Extension Center, Fayetteville, AR (AAREC Fayetteville). Each site was 17.7 to 35.4 km away from each other.

Lake Wedington unit of the OSFNF was created in the 1930s through the Northwest Arkansas Land Use Project that converted misused or idle farm land into recreational areas (Schalm 1973). From this location, five samples were collected from decaying logs (Table 4.1). Lee Creek area of OSFNF is located south of Devils Den State Park. It is primarily secondary growth forest that was also developed in the 1930s through efforts of the Civilian Conservation Corps (Smith 1992). Seven samples were collected at this location (Table 4.2). AAREC Fayetteville was established in 1888 under the Hatch Act of 1887, which provided research stations to land grant universities (Strausberg 1989, Hillison 1996). It contains mainly secondary pine forests that separate buildings and experimental plots. Thirteen samples (Table 4.3) were collected from both decaying logs and Sentricon® Termite Colony Elimination System bait stations (Dow AgroSciences, Indianapolis, IN, U.S.) containing untreated pieces of wood. Twenty-five samples representing the three species and three localities were examined in this study (Table 4.4).

All samples were collected into 95% ethanol and voucher specimens are housed at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, Arkansas, U.S. Morphological identification when possible used the keys of Scheffrahn and Su (1994) and

was confirmed genetically using PCR-RFLP (Szalanski et al. 2003) and DNA sequencing analysis using a region of the mtDNA 16S gene per Austin et al. (2004)

MITOCHONDRIAL DNA SEQUENCING

Genomic DNA was extracted from a head from an individual specimen using a salting-out procedure with in-house reagents (Sambrook et al. 2001). The 16S gene region of mtDNA was amplified using the primers 16S: LR-J-13007 (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati and Smith 1995) and LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3') (Simon et al. 1994). Consensus sequences were obtained from an alignment of sequences in both directions for each sample using Geneious v6.1.8 (Kearse et al. 2012). Haplotypes were determined by comparing to an in-house database (ALS unpublished).

MICROSATELLITE GENOTYPING

All specimens were confirmed to species using either PCR-RFLP analysis or a GenBank BLAST search using DNA sequences. Genomic DNA was extracted individually from the heads of 20 worker termites from each sample using a salting-out procedure with in-house reagents (Sambrook et al. 2001) and stored at -20°C. Twelve total microsatellite loci (*R. flavipes*: 8 loci; *R. hageni*: 11 loci; *R. virginicus*: 7 loci) (Vargo 2000, Dronnet et al. 2004) were amplified in a single multiplex of fluorescently-labeled primers (dye set: G5, Applied BioSystems, Life Technologies, Grand Island, NY, U.S.). Each species had a unique PCR reaction mix that can be found in Tables 4.5-4.7. The thermal cycler settings (modified from: Vargo 2000 and Tripodi 2014) consisted of a hot start at 95°C followed by a touchdown program of six cycles at 94°C (30s), 62°C (30s), and 72°C (30s) decreasing annealing temperature 1°C per cycle; then 30 cycles of 94°C (30s), 55°C (30s), and 72°C (30s); with a final extension at 72°C for 5min. Samples were genotyped at Iowa State University DNA Facility (Ames, IA, U.S.) using an ABI

Prism 3730 DNA Analyzer (Applied Biosystems, Life Technologies, Grand Island, NY, U.S.) with GeneScan 500 LIZ dye size standard (Applied Biosystems, Life Technologies, Grand Island, NY, U.S.).

MICROSATELLITE DATA ANALYSIS

Alleles were scored with the microsatellite plugin for Geneious v6.1.8 (Kearse et al. 2012). Samples and loci with more than 50% missing data were removed from analysis. To access if all samples represented unique colonies, pairwise F_{ST}-values were calculated with Arlequin v3.5.1.2 (Excoffier and Lischer 2010). According to Vargo (2003a), samples from unique colonies had significant F_{ST} p-values ($p<0.05$), whereas samples of the same colony yield non-significant F_{ST} p-values. For any repeatedly sampled colony, only the first sample obtained was used for further analysis.

Within each colony established from significant F_{ST} p-values, breeding structure was determined. Alleles per locus were calculated in Arlequin and were used to distinguish mixed family structure. A mixed family, defined by Deheer and Vargo (2004), contain more than four alleles at any one locus, a characteristic only possible with a colony containing two or more unrelated reproductives of the same sex. Remaining colonies that were not classified as mixed families were divided into simple and extended families based on if they significantly differ from Mendelian ratios (Vargo 2003a). Extended families were determined if there was a significant difference between the observed and expected heterozygosity of each locus, summed together in a G-test for goodness of fit in R v3.0.2 (R Core Team 2013). Simple families were determined by a non-significant value when comparing observed and expected heterozygosity.

RESULTS

MITOCHONDRIAL DNA

For *R. flavipes,* six of the eight samples had unique haplotypes. For *R. hageni* and *R. virginicus,* three of the eleven and six, respectively, were unique haplotypes. Within a species, haplotypes were not exclusive to geographic location collected. (Tables 4.1-4.3).

MICROSATELLITE DNA

A majority of pairwise F_{ST} values calculated for each species (Tables 4.8-4.10) were significant. When comparing *R. flavipes* samples, 6075 and 6076 sampled the same colonies as did sample 6082 and 6091 as indicated by the non-significant p-values in bold (Table 4.8). All *R. hageni* samples represent unique colonies (Table 4.9). For *R. virginicus*, many of the samples were from the same colony, with non-significant p-values (Table 4.10). To avoid the bias of oversampling a single colony, samples 6076, 6091, 416, 418, 419, and 691 were removed when comparing breeding structures between sites and species.

Two *R. flavipes* colonies were simple families (Table 4.11) with non-significant G-test values comparing observed and expected heterozygosity. The remaining six *R. flavipes* colonies were extended families. For *R. hageni* (Table 4.12), no colonies had more than four alleles, indicating no mixed families. Three *R. hageni* colonies also yielded non-significant G-test results for heterozygosity, indicating simple families. The remaining 7 colonies were extended families In *R. virginicus*, three loci were removed, yielding only four usable loci. However with only these four loci, two samples (415 and 6034) had more than four alleles per loci, indicating they are mixed families (Table 4.13).The remaining colonies were classified as extended families from the non-significant G-test.

DISCUSSION

As inferred from the F_{ST} tables, most samples represent unique colonies. There are several instances, however, where the same colony was sampled multiple times. Samples 6075 and 6076 were collected from Sentricon® Termite Colony Elimination System bait stations within several meters of each other at AAREC Fayetteville and both sampled the same colony. Because they are near each other, this is not unexpected; however, it is interesting that only these two bait stations were foraged on by this colony, as there were multiple other bait stations in the same area that contained termites from different colonies. Samples 6082 and 6091 also represent the same colony and were collected within several meters from each other at Lee Creek area of OSFNF from fallen logs. Samples 415, 416, 418, 419, and 691 were also collected from closely spaced Sentricon® Termite Colony Elimination System bait stations in a different area of AAREC Fayetteville. These are also understandably the same colony due to the close proximity of sampling. Overall, the basic spatial dynamics shown from these examples highlight an interesting aspect of termite biology that could be further investigated in future research.

This is the first study to examine both the mitochondrial DNA haplotypes and microsatellite DNA genotypes. From this pairing, we observed that within a colony, multiple mitochondrial haplotypes may exist. This is a novel occurrence that requires more research into the mechanism of this characteristic.

In these three species of *Reticulitermes* in northwest Arkansas, only 22% of the colonies sampled were simple families, 72% were extended families, and 6% (1 sample) was a mixed family (Table 4.14). While the sample size for each site and species are too small to adequately describe the proportions of breeding systems in the area, they do provide some insight. From these 18 samples, *R. flavipes* and *R. virginicus* colonies contain approximately the same ratio of simple to extended family. *R. hageni* was divided with 1 simple families and 1 mixed families

(Table 4.14) but require further samples and loci. Compared to previous studies, the percent of extended families in this study is higher (Vargo and Husseneder 2009). This also is the first record of mixed families in *R. hageni* anywhere in the U.S. Overall, the results obtained in northwest Arkansas are similar to those found in the eastern U.S. Further sampling could confirm that the unique aspects of this study are a result of the location rather than the small sample size.

ACKNOWLEDGEMENT

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TABLE 4.1. Termite samples from Lake Wedington unit of OSFNF.

Lab ID#	Species	16S Haplotype	Coordinate	Date Collected
6033	*R. hageni*	H1	36.131554, -94.391383	2 July 2014
6034	*R. virginicus*	V1	36.131276, -94.394598	2 July 2014
6035	*R. hageni*	H1	36.132515, -94.395116	2 July 2014
6037	*R. hageni*	H1	36.131405, -94.394698	2 July 2014
6038	*R. hageni*	H4	36.131937, -94.394154	2 July 2014

TABLE 4.2. Termite samples from Lee Creek area of OSFNF.

Lab ID#	Species	16S Haplotype	Coordinate	Date Collected
6082	*R. flavipes*	R	35.761857291667, -94.273830208333	12 October 2014
6091	*R. flavipes*	GG, SS	35.761834375, -94.273817708333	12 October 2014
6092	*R. hageni*	H1	35.761713666667, -94.273759333333	12 October 2014
6095	*R. flavipes*	TT	36.022323125, -94.219738333333	12 October 2014
6096	*R. hageni*	H1	36.039762, -94.220402666667	12 October 2014
6097	*R. flavipes*	M, QQ, F3	36.039741481481, -94.219918074074	12 October 2014

TABLE 4.3. Termite samples from AAREC Fayetteville.

Lab ID#	Species	16S Haplotype	Coordinate	Date Collected
414	*R. hageni*	H1	N.A.	2003
415	*R. virginicus*	V1	N.A.	2003
416	*R. virginicus*	V5	N.A.	2003
418	*R. virginicus*	V5	N.A.	2003
419	*R. virginicus*	V1	N.A.	2003
691	*R. virginicus*	V3	N.A.	2003
6075	*R. flavipes*	TT	36.099075769231, -94.165908525641	24 September 2014
6076	*R. flavipes*	TT	36.098942594697, -94.165817746212	24 September 2014
6077	*R. flavipes*	TT	36.098944713262, -94.165818799283	24 September 2014
6079	*R. hageni*	H1	36.099043842593, -94.165938935185	24 September 2014
6080	*R. hageni*	H2	36.099044866667, -94.165906666667	24 September 2014
6081	*R. hageni*	H1	36.099223333333, -94.165998541667	24 September 2014
6099	*R. flavipes*	SS, GG	N.A.	21 January 2015

TABLE 4.4. Number of termite samples by location.

Site	*R. flavipes*	*R. hageni*	*R. virginicus*	Total
Lake Wedington unit of OSFNF	0	4	1	5
Lee Creek area of OSFNF	4	2	0	6
AAREC Fayetteville	4	4	5	13
Totals	8	10	6	24

TABLE 4.5. Primer dyes and concentrations for *R. flavipes*.

Primer	Citation	Dye	Amount per sample (µl)
RS13	Dronnet et al. 2004	FAM	0.42
RS33	Dronnet et al. 2004	FAM	0.30
RS62	Dronnet et al. 2004	VIC	0.25
RF 15-2	Vargo 2000	FAM	0.22
RS43	Dronnet et al. 2004	NED	0.19
RS16	Dronnet et al. 2004	NED	0.14
RF 6-1	Vargo 2000	NED	0.11
RS10	Dronnet et al. 2004	VIC	0.06

Additional PCR ingredients:
2.5 µl Buffer 5X Promega
0.7 µl $MgCl_2$ 25mM
0.75 µl dNTP
3.07 µl H_2O
0.1 µl Taq Poymerase
2.0 µl DNA

TABLE 4.6. Primer dyes and concentrations for *R. hageni*.

Primer	Citation	Dye	Amount per sample (µl)
RS13	Dronnet et al. 2004	FAM	0.46
RS16	Dronnet et al. 2004	NED	0.38
RS43	Dronnet et al. 2004	NED	0.36
RS33	Dronnet et al. 2004	FAM	0.27
RF 15-2	Vargo 2000	FAM	0.25
RF 1-3	Vargo 2000	VIC	0.25
RF 11-2	Vargo 2000	VIC	0.24
RS78	Dronnet et al. 2004	PET	0.15
RF 6-1	Vargo 2000	NED	0.13
RS10	Dronnet et al. 2004	VIC	0.11
RF 24-2	Vargo 2000	FAM	0.01

Additional PCR ingredients:
2.5 µl Buffer 5X Promega
0.7 µl $MgCl_2$ 25mM
0.75 µl dNTP
1.23 µl H_2O
0.1 µl Taq Poymerase
2.0 µl DNA

TABLE 4.7. Primer dyes and concentrations for *R. virginicus*.

Primer	Citation	Dye	Amount per sample (µl)
RS13	Dronnet et al. 2004	FAM	0.41
RS16	Dronnet et al. 2004	NED	0.29
RS33	Dronnet et al. 2004	FAM	0.21
RS43	Dronnet et al. 2004	NED	0.20
RF 11-2	Vargo 2000	VIC	0.18
RF 6-1	Vargo 2000	NED	0.07
RS10	Dronnet et al. 2004	VIC	0.04

Additional PCR ingredients:
2.5 µl Buffer 5X Promega
0.7 µl $MgCl_2$ 25mM
0.75 µl dNTP
3.65 µl H_2O
0.1 µl Taq Poymerase
2.0 µl DNA

TABLE 4.8. F_{ST} table for *R. flavipes* (p-values). **Bold** indicates not significant ($p>0.05$), i.e. same colony.

Sample/Sample	6075	6076	6077	6082	6091	6095	6097	6099
6075	*							
6076	**0.42342**	*						
6077	0.00000	0.02703	*					
6082	0.00000	0.00000	0.00000	*				
6091	0.00000	0.00000	0.00000	**0.08108**	*			
6095	0.00000	0.00000	0.00000	0.00000	0.00000	*		
6097	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	*	
6099	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	*

TABLE 4.9. F_{ST} table for *R. hageni* (p-values). **Bold** indicates not significant ($p>0.05$), i.e. same colony.

Sample/ Sample	414	6033	6035	6037	6038	6079	6080	6081	6092	6094	6096
414	*										
6033	0.00000	*									
6035	0.00000	0.00000	*								
6037	0.00000	0.00000	0.00000	*							
6038	0.00000	0.00000	0.00000	0.00000	*						
6079	0.00000	0.00000	0.00000	0.00000	0.00000	*					
6080	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	*				
6081	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	*			
6092	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	*		
6094	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	*	
6096	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	*

TABLE 4.10. F_{ST} table for *R. virginicus* (p-values). **Bold** indicates not significant ($p>0.05$), i.e. same colony.

Sample/Sample	415	416	418	419	691	6034
415	*					
416	**0.16453**	*				
418	0.00100	0.00112	*			
419	**0.06005**	**0.07042**	**0.13472**	*		
691	**0.28795**	**0.34843**	0.00000	**0.08180**	*	
6034	0.00000	0.00000	0.00000	0.00000	0.00000	*

TABLE 4.11. Microsatellite loci variability for *R. flavipes*.

Colony	Family Structure	Alleles per locus							Mean alleles	G-test for observed and expected heterozygosity
		RF 15-2	RS33	RS13	RS10	RS62	RS43	RS16		
6075	extended	1	2	2	3	2	1	1	1.7143	G_{tot}=77.65, df=8, p<0.001
6076	simple	2	2	2	2	2	1	3	2.0000	G_{tot}=13.66, df=8, p=0.091
6077	extended	1	2	2	2	2	1	1	1.5714	G_{tot}=26.51, df=8, p<0.001
6082	extended	1	1	3	3	2	1	2	1.8571	G_{tot}=15.71, df=8, p=0.047
6091	extended	2	2	2	2	2	1	1	1.7143	G_{tot}=34.38, df=8, p<0.001
6095	simple	1	1	2	2	1	2	1	1.4286	G_{tot}=10.84, df=8, p=0.211
6097	extended	1	2	1	3	1	1	1	1.4286	G_{tot}=45.25, df=8, p<0.001
6099	extended	1	2	2	2	1	1	1	1.4286	G_{tot}=23.99, df=8, p=0.007

TABLE 4.12. Microsatellite loci variability for *R. hageni*.

Colony	Family Structure	Alleles per locus										Mean alleles	G-test for observed and expected heterozygosity
		RF 15-2	RS33	RS13	RS10	RF 1-3	RF 11-2	RF 6-1	RS43	RS16	RS78		
414	extended	3	1	3	1	3	1	4	3	3	4	2.6000	G_{tot}=38.20, df=10, p<0.001
6033	extended	4	1	2	2	2	1	3	2	1	3	2.1000	G_{tot}=64.97, df=10, p<0.001
6035	extended	4	1	1	3	1	1	4	2	1	3	2.1000	G_{tot}=28.88, df=10, p=0.001
6037	extended	2	1	1	2	1	1	4	2	2	2	1.8000	G_{tot}=19.40, df=10, p=0.035
6038	simple	2	1	1	3	1	1	3	4	1	3	2.0000	G_{tot}=13.55, df=10, p=0.195
6079	extended	3	1	2	4	3	1	2	2	4	2	2.4000	G_{tot}=115.77, df=10, p<0.001
6080	simple	2	1	1	2	1	1	3	1	2	3	1.7000	G_{tot}=10.14, df=10, p=0.428
6081	extended	2	1	2	4	2	1	3	3	1	3	2.2000	G_{tot}=99.58, df=10, p<0.001
6092	extended	2	1	2	3	2	1	2	2	0	1	1.6000	G_{tot}=20.74, df=10, p=0.014
6096	simple	2	1	2	1	2	1	3	2	2	2	1.8000	G_{tot}=7.58, df=10, p=0.670

TABLE 4.13. Microsatellite loci variability for *R. virginicus*.

Colony	Family Structure	Alleles per locus				Mean alleles	G-test for observed and expected heterozygosity
		RS33	RS13	RS43	RS16		
415	mixed	2	**5**	2	**5**	2.5714	NA
416	extended	3	3	2	2	2.1429	$G_{tot}=41.71$, df=4, $p<0.001$
418	extended	2	4	3	4	3.1429	$G_{tot}=20.85$, df=4, $p<0.001$
419	extended	2	4	1	2	2.1429	$G_{tot}=17.88$, df=4, $p=0.001$
691	mixed	2	**5**	3	1	2.4286	NA
6034	extended	1	3	1	2	2.5714	$G_{tot}=57.14$, df=4, $p<0.001$

TABLE 4.14. Summary of family structure split by species and location.

Site	Simple Families	Extended Families	Mixed Families
Lake Wedington	20% (1)	80% (4)	0%
R. flavipes	0%	0%	0%
R. hageni	25% (1)	75% (3)	0%
R. virginicus	0%	100% (1)	0%
Lee Creek	40% (2)	60% (3)	0%
R. flavipes	33% (1)	67% (2)	0%
R. hageni	50% (1)	50% (1)	0%
R. virginicus	0%	0%	0%
AAREC Fayetteville	13% (1)	75% (6)	13% (1)
R. flavipes	0%	100% (3)	0%
R. hageni	25% (1)	75% (3)	0%
R. virginicus	0%	0%	100% (1)
Divided by species:			
R. flavipes	17% (1)	83% (5)	0%
R. hageni	30% (3)	70% (7)	0%
R. virginicus	0%	50% (1)	50% (1)
Totals	22% (4)	72% (13)	6% (1)

FIGURE 4.1. Sample sites in northwest Arkansas: Lake Wedington unit of OSFNF, AAREC Fayetteville, Lee Creek area of OSFNF. Red bar equals 8 km.

CHAPTER 5

CONCLUSION

Subterranean termites are an economically significant pest that damage many houses in the U.S. each year. In this research, molecular genetic tools were used to investigate various aspects of termite identification and biology. While these projects simply scratch the surface of the potential applications of molecular genetics to study termites, they serve as examples and a base for future work.

The first project, developing a multiplex PCR protocol to distinguish the invasive Formosan subterranean termite from other subterranean termites, is important as a monitoring tool for the spread of this invasive species. This enables quick screening (approx. 12hrs total) of any sample, not relying on the descriptive castes with uncertain morphological identifications. This tool is simple, distinguishing one species from all others, but these same tools could be applied to identification of other invasive termites, or to aid in the identification of morphologically cryptic species.

Next, a new, isolated species of *Reticulitermes* was described from southern Mississippi. In this project, the morphological ambiguity required molecular genetic techniques to identify this species. This also introduces the possibility that isolated species are unnoticed in the U.S. due to inability for morphological identification.

Finally, this thesis applied previously developed molecular tools to investigate the breeding structure of *Reticulitermes* colonies in Arkansas. While this was not novel, but rather an expansion of the examined geographical range, this had important discoveries including the first

observation of a *R. hageni* mixed family as well as the first observation of a termite colony containing multiple mtDNA haplotypes. Further sampling can only expand and strengthen these findings.

Overall, molecular genetics have nearly endless possibilities for their applications to the study of termites. These contributions provide the foundation for future work that is needed to better understand this important economic pest.