11-2018

Population Genetics of the Eastern Subterranean Termite, Reticulitermes flavipes, in the Southern Appalachian Mountains

Lee Ann Passarella

Population genetics of the eastern subterranean termite, *Reticulitermes flavipes*, in the southern Appalachian Mountains

by Lee Ann Passarella

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2019

Approved By

Advisor: Dr. Ryan Garrrick

Reader: Dr. Rebecca Symula

Reader: Dr. John Samonds

ACKNOWLEDGEMENTS

I would like to extend my greatest gratitude to Dr. Ryan Garrick for being my mentor for the past three and a half years in the laboratory. Working with him has challenged me in ways no one has before and made me realize my full potential as a human. I would like to extend a special thank you to John Banusiewicz for first getting me involved with lab research and inspiring me to take on my own research project. I would like to thank all members of the Garrick lab for always celebrating my successes and being my friends throughout the years.

ABSTRACT

LEE ANN PASSARELLA: Population genetics of the eastern subterranean termite, *Reticulitermes flavipes*, in the southern Appalachian Mountains
(Under the direction of Dr. Ryan Garrick)

As an area rich in biodiversity, the Appalachian Mountains are an ideal place for biological research. The pronounced impacts of Pleistocene climatic cycles on forest distributions over time have shaped the evolutionary history of animals that depend on these habitats, underpinning the research that is presented here. The present study on the eastern subterranean termite, *Reticulitermes flavipes,* was conducted to better understand the spatial distribution of genetic diversity within and among populations of this species in the context of the aforementioned historical climatic changes in the southern Appalachian Mountain region. I hypothesized that there would be geographically localized, distinct genetic groups due to the limited dispersal ability of *R. flavipes*, coupled with the long history of repeated habitat fragmentation during the Last Glacial Maximum and earlier. This study used termite samples collected throughout the southern Appalachians and extracting their DNA. Next, mitochondrial gene regions, cytochrome c oxidase subunit I (COI) and cytochrome c oxidase subunit II (COII), and the nuclear gene region, nuclear endo-beta-1,4-glucanase (EB14G), were amplified using polymerase chain reaction (PCR). Gel electrophoresis was then used to confirm amplification success, and PCR products were sequenced at an external sequencing facility. After editing and aligning those sequences, a phylogenetic tree was estimated from the mitochondrial data, and natural genetic groups were identified and mapped according to the spatial data associated with them. The phylogenetic tree supported four distinct monophyletic groups, all of which were spatially clustered together in geographic space

(i.e., there was phylogeographic structure). To determine if patterns of phylogeographic structure identified here truly reflect the history of the species as a whole, *R. flavipes* individuals in areas that were not well represented in the present study, along with the amplification and screening of nuclear loci will be necessary.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION

Phylogeography

Phylogeography is a field of research that focuses on reconstructing the long-term population history of a species. Phylogeographic studies can answer questions about population expansions, locations of past habitat refugia, the extent of gene flow, and the sequence of events that produced the geographic distributions of genetic variation within species (Emerson & Hewitt 2005). To achieve this, molecular data are used to estimate a genealogy (i.e., evolutionary relationships), which is interpreted by linking genetic diversity to the spatial distribution of the species of interest. The resulting inferences about evolutionary history can shed light on past events that impacted the genetic diversity of a species since the most recent glacial climatic period and earlier (Emerson & Hewitt 2005). Phylogeography of animal species is mainly assessed through mitochondrial DNA (mtDNA). Nuclear DNA data can accompany mtDNA data to provide more accurate gene flow data, and to better estimate phylogenetic relationships. Phylogeographic studies often focus on areas of great biodiversity because Pleistocene climatic changes, or the formation of barriers (e.g., mountains, or rivers), had widespread effects on differentiation of many populations and species located in those areas (Rissler & Smith 2010). The present research focuses on the biologically diverse southern Appalachian Mountains.

The southern Appalachian Mountains

The Appalachian mountain range spans 13 states from Alabama and Georgia up to Maine, extending over 1,000 miles. There are six ecoregions in the mountain range (Figure 1) that make up the diverse ecosystem that is the Appalachian Mountains. The Appalachians are known for their high biodiversity and geological stability over the past 450 million years. Regarded as the largest continuous center of endemism in the world, the Appalachian Mountains are home to over 6,300 plant species and over 700 animal

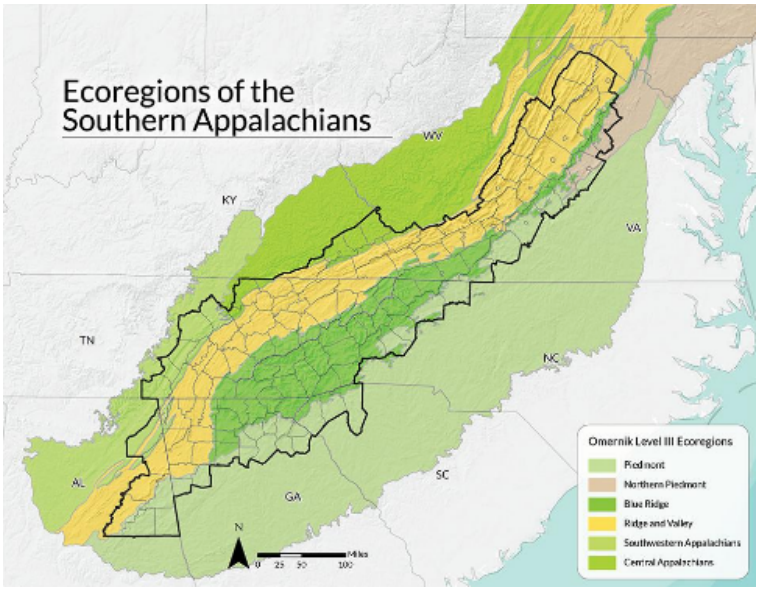

Figure 1. The ecoregions of the Appalachian mountain range spanning from Alabama and Georgia up to West Virginia/Virginia. The different colors represent the six physiographic regions of the Appalachian Mountains (source:http://southernappalachianvitalityindex.org /ecoregions-southern-appalachians).

species. They contain the highest concentration of salamanders in the world and are rich with a diverse set of tree species (Davis 1983). Temperatures in the summer rarely surpass 32 degrees Celsius, with considerable rainfall during those months. Winter brings rougher and colder weather ranging from -1 to 4 degrees Celsius temperatures (http://southernappalachianvitalityindex.org/air-and-water/climate). The Appalachians are considered to be a "cooling island" in the southern area because the region carries colder temperatures than the surrounding areas outside the mountain range (Oregon State University PRISM Climate Group 2011). Because of this, the southern Appalachians are recognized as a refugial area for many species in the region that do not readily tolerate warmer temperatures. The northern parts of the Appalachians have comparatively lower biodiversity because of the colder weather present in the north.

Environmental changes such as the past glacial/interglacial cycles have impacted the spatial distribution of genetic diversity within species (Delcourt & Delcourt 1998). These 100,000 year glacial and 10,000 year interglacial cycles occurred repeatedly throughout the Quaternary period, which dates back 2.5 million years ago. The rotations were cool and dry glacial periods and that alternated with hot and humid interglacial cycles. During the most recent glacial period, the Laurentide Ice Sheet (Figure 2) covered most parts of the northern Appalachians. Not able to survive the colder, higher altitudes, trees flourished in the lower valleys and ravines of the mountain range, where those trees helped forest-dependent organisms thrive. The glacial periods strongly contributed to fragmenting the distribution of forests, which in turn caused isolation among populations of species residing in the depths of the Appalachians. As for the hot and humid interglacial periods, forest distributions expanded upslope and reconnected previously

fragmented and isolated refugial areas. As a consequence, forest dependent organisms recolonized mountain tops, and may have reconnected with other populations. Overall, the glacial and interglacial cycles contributed to the spatial distribution of genetic diversity within and among species in the Appalachians (Davis 1983). The southern Appalachians are considered to have acted as a refuge during the Pleistocene because the region was not covered by the Laurentide ice sheet; traces of glacial ice were only detected above the Mason-Dixon line. The southern Appalachians remained free from the

Figure 2. Map displaying the spatial extent of the Laurentide Ice Sheet in North America during the Last Glacial Maximum (source:https://imnh.iri.isu.edu/digitalatlas/geog/native/text/history.htm).

ice sheet, so many species thrived there. Following the Last Glacial Maximum around 15,000 years ago, the ice sheet in the northern parts of the Appalachians melted away within just 2,000 years, giving way to newly habitable areas, and as a result, a northward

redistribution of species occurred (Williams et al. 2004). Today, northern species have relatively low genetic diversity compared to the species in the southern Appalachians because of the late post-glacial migration up into the now ecologically stable North (Walker et al. 2009). Released from the refugium in the south, species could now survive the climate in the north (Delcourt & Delcourt 1998). Upland adapted species are often sensitive to warmer temperatures (and associated desiccation risk) that occur in lowlands/valleys separating montane areas. Because of the difficulty traversing these inhospitable areas, only species with strong dispersal capabilities are readily able to move between isolated habitat patches. Thus, species with limited dispersal capabilities are likely to have been impacted the most by the Pleistocene glacial cycles. Termites are the focus of the work presented here, and they generally have limited dispersal capabilities.

The eastern subterranean termite, *Reticulitermes flavipes*

In natural settings, termites contribute immensely to forest health. By contributing to the decomposition of dead wood, nitrogen is recycled into the air quickly, and therefore termites indirectly make a forest more productive (Ulyshen 2016). Although termites are important to the environment around them, they are considered to be the most expensive pest, with the damage they cause to human-made structures and the associated control measures costing billions of dollars annually. The eastern subterranean termite, *Reticulitermes flavipes*, is one of the most common termite species in the eastern United States. It is considered to be the most economically important wood-eating insect in the United States (www.wikipedia.org/wiki/Eastern_subterranean_termite). *R. flavipes* is common in the southern Appalachians but can be found in northern regions as well.

With populations consisting of huge numbers of individuals, termites live in "shelter tubes" (seen in Figure 3, Panel B) within dead wood, feeding on the cellulose. *R. flavipes* colonies consist of three castes: workers (seen in Figure 3, Panel A), soldiers, and kings/queens. On warm and wet days, alates (i.e., individuals with wings) will "swarm" to find a mate and establish a nesting site (Vargo & Husseneder 2009). Their dispersal capabilities are limited to the colony itself, but a queen will lay up to 30,000 eggs in a day.

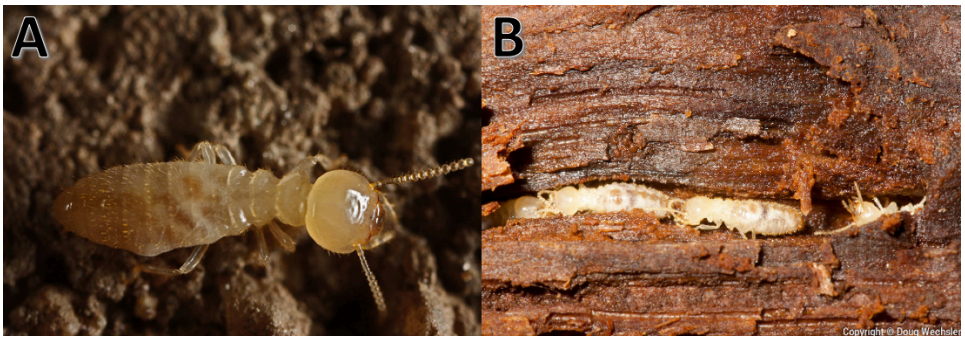

Figure 3. Pictures of the termite species *Reticulitermes flavipes*.
Panel A: Termite of the worker caste.
Panel B: Termites in their "shelter tubes" of dead wood.
Source for Panel A: http://ninnescahlife.wichita.edu/node/414
Source for Panel B:
https://dougw.photoshelter.com/image/I00000A8dL22kno4

Goals

The present study on the eastern subterranean termite, *Reticulitermes flavipes,* was conducted to better understand the spatial distribution of genetic diversity within this species located in the southern Appalachian Mountains, in the context of historical climatic changes that occurred in the region. Since dispersal ability of *R. flavipes* is limited, genetic signatures of past forest fragmentation during the Last Glacial Maximum

and earlier should still be detectable today. An understanding of the phylogeography of this species will contribute to ongoing studies of other forest invertebrates in the region.

METHODS

Geographic sampling

Between 2012 and 2017, *R. flavipes* termites were sampled by members of the Garrick lab from 30 intermediate- to late-stage rotting logs across 7 states within the southern Appalachian Mountains and surrounding forested areas. All specimens were geo-referenced using a handheld GPS, and stored in 95% ethanol. Collection location information is given in Table 1.

Table 1: Geographic locations from which *Reticulitermes flavipes* termites were sampled. Each site has a unique ID, and GPS coordinates are reported in decimal degrees.

Site ID	State	Longitude	Latitude
A03	GA	-84.63805	34.77972
A04	GA	-85.06536	34.57297
A09	GA	-85.24268	34.56515
A13	AL	-85.70074	33.56059
A14	AL	-85.81731	33.46215
A16	AL	-86.07201	33.2015
A18	GA	-84.71137	34.87866
A21	GA	-84.3388	34.77507
A22b	GA	-84.25093	34.68311
A30	TN	-83.51849	35.65682
A32	NC	-83.31077	35.52117
A37	TN	-83.21343	35.7714
A40	GA	-84.6914	34.75931

A56	VA	-78.78368	38.12902
A64	VA	-78.3406	38.62592
A70b	VA	-79.3498	38.04052
A73	TN	-87.52677	35.39384
A92b	NC	-83.59187	35.32969
A97	NC	-82.48742	35.59535
A107	WV	-79.38506	38.82585
A108b	WV	-79.48494	38.72694
A124	AL	-85.87318	33.40451
A131b	AL	-87.33273	34.41979
A134	AL	-85.58357	34.4554
A137	AL	-85.4573	33.9634
A139	GA	-85.26428	34.1226
A141	SC	-83.10755	34.862
A143	SC	-83.08929	34.94523
A145b	SC	-83.22783	34.72782
A146b	SC	-83.31242	34.77755

DNA Extraction

DNA was extracted from the entire body of each termite, with only members of the worker caste used for isolating DNA. Extraction was completed using Qiagen DNeasy Blood & Tissue kit, following the manufacturer's recommendations (see Purification of Total DNA from Animal Tissues, Spin-Column Protocol, available online at https://www.qiagen.com). This protocol was followed step-by-step with the following four exceptions. First, before beginning the extraction from the termite body, I allowed

the ethanol, in which the termite was preserved, to fully evaporate from the animal. The ethanol evaporated for at least 45 minutes on a Kimwipe under a petri dish to prevent sample contamination. In step one, I manually crushed individual termite samples with a pestle, using a new pestle for each sample. Second, in step two, I added 4 microliters (μL) of RNase A (100 mg/mL) to the sample following overnight incubation at 56°C. I mixed the RNase A by vortexing and incubated the samples for two minutes at room temperature. Third, in step six, to ensure that no ethanol carried over into the next step, I emptied the collection tube to reuse it in another centrifugation for one minute at 20,000 x g (14,000 rpm). Finally, in steps seven and eight, instead of eluting the samples with 100 μL, I performed two elutions using 65 μL of Buffer AE, resulting in a total of 130 μL of extracted DNA.

Polymerase Chain Reaction (PCR)

After extracting DNA from termite samples, mitochondrial DNA and nuclear DNA target gene regions were amplified via PCR. I amplified two mitochondrial genes, cytochrome c oxidase subunit I (COI) and subunit II (COII). We targeted these two genes because they have fast DNA sequence mutation rates, which contribute to determining the genetic variation of species. I also amplified a nuclear DNA gene, nuclear endo-beta-1,4-glucanase (EB14G). For PCR amplification, termite DNA samples were added to a tube with a master mix of PCR ingredients. Listed components of the master mix are located in Table 2. Forward (F-) and reverse (R-) primers differed to amplify target genes in the samples; those primers for certain target gene regions are located in Table 3. After adding the master mix to a 1:19 dilution of termite DNA, the PCR samples were mixed

via vortexing and then briefly centrifuged. Thermal cycling conditions used for the amplification of all target gene regions are listed in Table 4.

Table 2: Standard Master Mix (15 µL)

Component (concentration)	**Volume (µL)**
5x Buffer	3.00
$MgCl_2$ (25 mM)	1.20
dNTPs (1.25 mM)	2.40
BSA (10 mg/mL)	0.75
dH_2O	4.50
Forward-primer (10 mM)	0.75
Reverse-primer (10 mM)	0.75
Go-Taq (5u/uL)	0.15
DNA (1:19 dilution)	1.50

Table 3: Target genes and PCR primers used to amplify them via PCR. Abbreviations: mitochondrial DNA (mtDNA), nuclear DNA (nDNA)

Target gene	Primer name	Primer sequence (5' to 3')	Original reference
mtDNA COI	LCO-1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO-2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
mtDNA COII	CO2-forward	AGAGCWTCACCTATTATAGAAC	Park et al. (2004)
	TK-N-3785	GTTTAAGAGACCAGTACTTG	Simon et al. (1994)
nDNA EB14G	Ret_EB14G_F	ATGGAGGTCGCAGCTACGTC	Hyseni & Garrick (in review)
	Ret_EB14G_R	GGCGCTGTTGTACGTGTTCCAG	Hyseni & Garrick (in review)

Table 4: PCR thermal cycling conditions.

Step	Temperature (℃)	Duration	Number of Cycles
Denaturation	95	2 minutes	1
Denaturation	95	30 seconds	35
Annealing	52	30 seconds	
Extension	72	1 minute	
Extension	72	2 minutes	1
Hold	12	Indefinite	

Agarose Gel Electrophoresis

Following PCR, agarose gel electrophoresis was used to estimate the length of the amplified products via comparison to a DNA ladder. The specific base pair lengths were used to confirm successful PCR amplification of each targeted gene region, and also to confirm that there was no contamination. I poured and set gels using 1.5% agarose gel with Gel Red, into which I loaded my samples (seen in Figure 4). When my gel was set, I loaded 5 μL of each PCR sample into the gel wells. I included a 100 base pair (bp) ladder in the far-left lane (Lane 1) and a negative control in the last lane (Lane 14) for comparison. Times for running the gels ranged from 1-2 hours, running on a range of 80-120 volts. After running the gels, bright bands (seen in Figure 4), which referenced the fragment size of the DNA sequence, were expected to be 750 bp long for the COI and COII genes and 500 bp for the nuclear gene.

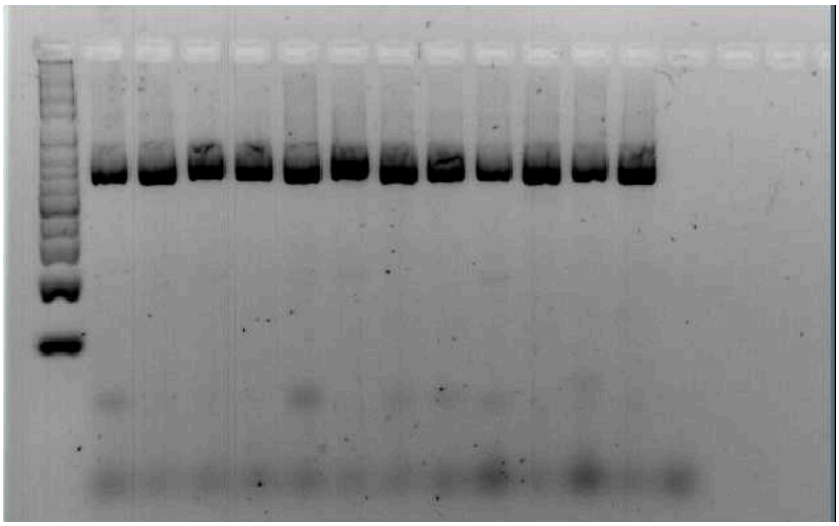

Figure 4. Resulting gel from amplification of COII mitochondrial gene. Lane 1 contains a 100bp ladder. Termite DNA samples that were amplified for the COII gene were put in lanes 2-13. The dark bands are at an expected 750 bp. Lane 14 acted as a negative control and indicated no signs of contamination.

ExoSAP, DNA sequencing, & Sequence Editing

Following the manufacturer's recommended protocol, PCR products were purified using ExoSAP-IT (Affymetrix), and then sequenced at Yale University's DNA Analysis Facility on Science Hill. The program used for sequence editing was Molecular Evolutionary Genetics Analysis (MEGA) v6.06 (Tamura et al. 2013). For each individual termite, the COI and COII sequences were concatenated (i.e., joined together) for analyses purposes, given that these two mtDNA genes are inherited as a single unit. When first editing the sequences, the ends were trimmed so that only high-quality sequences were used for downstream analyses. Editing the remaining sequences was as follows. Incorrectly named bases were edited to reflect the correct nucleotide character (i.e., A, T, C, or G). Wherever a chromatogram peak was of very low intensity, that base was reported as "n" to indicate an ambiguous character state.

Analyses

Although I worked with over 60 samples, my analyses consisted of only the 30 termites listed in Table 1. These 30 termites were chosen because each of these samples had little or no missing data (i.e., full length COI and COII sequences, with very few ambiguous base calls), and together they represented most of the geographic area of the Southern Appalachians that had been sampled. The total length of concatenated DNA sequence alignment, and the number of variable sites contained within it, was calculated using MEGA.

Phylogenetic tree estimation and geographic mapping

A phylogenetic tree was inferred using the Neighbor-joining method (Saitou & Nei 1987), in MEGA. Uncorrected p-distance genetic distance measures were used, and 1,000 bootstrap replicates were run to assess node support (Felsenstein 1985). Once a tree had been estimated, natural genetic groups were identified based on the following steps: (1) identify nodes with strong bootstrap support (≥ 75%); and (2) starting from the tips of the tree, move back towards the root and identify the most inclusive group of sequences that form a clade with strong support. The spatial distributions of these natural genetic groups identified from the phylogenetic tree were then plotted using the GPS coordinates of each individual member. This was done in Google Maps.

Assessment of levels of genetic diversity

To evaluate genetic difference among groups identified from the phylogenetic tree, mean uncorrected p-distances between all pairs of groups were calculated in MEGA. To assess levels of genetic variation within groups, mean uncorrected p-distances were calculated among sequences that reside within each group. Although sample sizes within groups varied, mean uncorrected p-distances should not be affected by this

RESULTS

After concatenation, the total length of the COI+COII mtDNA sequence alignment was 1,117 bp. There were 72 variable sites contained within this alignment.

Phylogenetic tree estimation and geographic mapping

I was able to identify four distinct groups (seen in Figure 5, Panel B); 3 of these groups have strong support values and contain multiple sequences, while the fourth yellow group only contains a single sequence. The blue and green groups appear to share a most recent common ancestor (i.e., they are sister groups). The red group is the most divergent of the four. The groups appear to be spatially distributed according to the phylogenetic tree. As seen in Figure 5 Panel A, the colored groups (i.e., red, blue, green

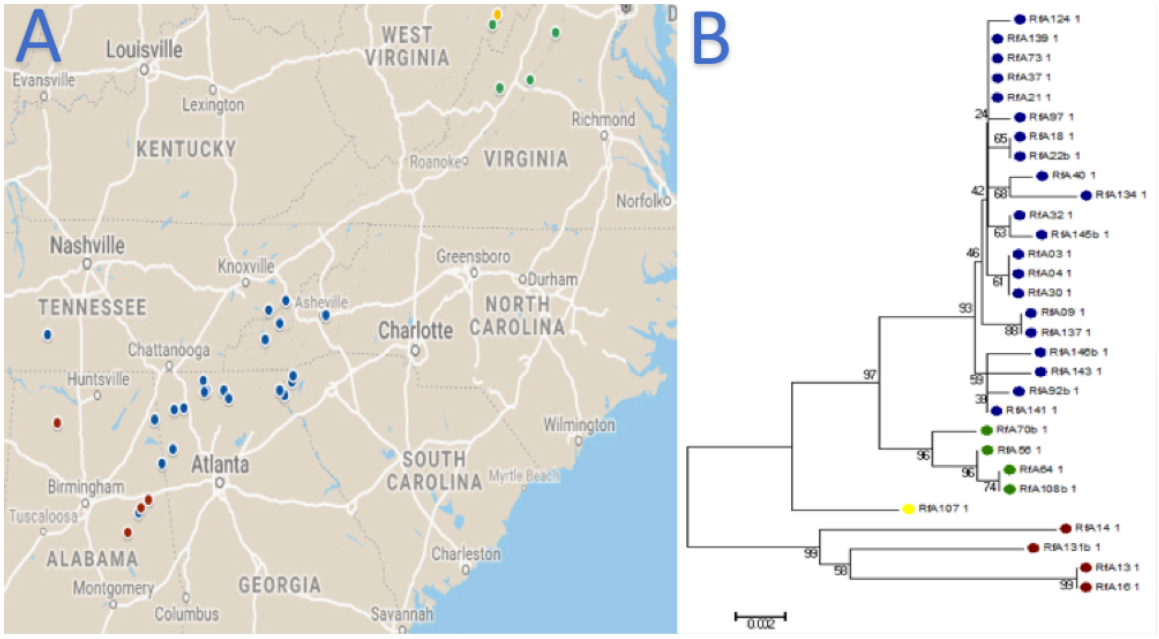

Figure 5. Geographic distribution and evolutionary relationships of DNA sequence haplotypes. Panel A: map displaying the spatial distribution of 30 *Reticulitermes flavipes* samples. Panel B: phylogenetic tree constructed from the DNA sequences from 30 *R. flavipes* samples. Determined taxa from a bootstrap value ≥75% then grouped into respective colors: blue, green, yellow, and red.

and yellow) all appear to be clustered in the same geographic regions. The red group is clustered solely in Alabama. The green group is present in the north in West Virginia and Virginia, while the yellow group is only located in West Virginia. The blue group is scattered throughout the southern parts of the Appalachians, states including Alabama, Georgia, Tennessee, North Carolina, and South Carolina. Notably, the blue and green group have a sister relationship, but they are contained in different geographic regions.

Assessment of levels of genetic diversity

Table 5 shows the between-group comparisons that were calculated for all pairs of genetic groups. The highest divergence was seen between the red/blue groups and the red/green groups with a mean uncorrected p-distance of 0.028. The comparison between the green and blue group was the least divergent, probably because they share a common ancestor with each other more recently than they do with any other groups. Table 6 shows the pairwise comparisons within groups. The red group has the highest diversity, and the blue and green groups have lower diversities. The value for the yellow group could not be calculated because there is only one DNA sequence haplotype.

Table 5: Values for the between group pairwise comparisons of the data set.

	BLUE	**RED**	**GREEN**	**YELLOW**
BLUE	**-**	**-**	**-**	**-**
RED	**0.028**	**-**	**-**	**-**
GREEN	**0.010**	**0.028**	**-**	**-**
YELLOW	**0.013**	**0.024**	**0.012**	**-**

Table 6: Values for the within group pairwise comparisons of the data set.

BLUE	**0.002**
RED	**0.015**
GREEN	**0.002**
YELLOW	**-**

DISCUSSION

The present research identified four genetic groups in the termite species, *Reticulitermes flavipes,* within the southern Appalachian Mountains. Furthermore, members of each of the four groups showed strong clustering in geographic space. When there is a close relationship between phylogeny and geography within species, referred to as "phylogeographic structure," it is often attributed to historical events such as Pleistocene climate cycles (Emerson & Hewitt 2005). Because these events could have had similar effects on other forest invertebrate species in the southern Appalachians, the findings for termites were compared to other related phylogeographic studies in the region.

Other related studies

There are similarities in the resulting phylogeographic structure of *R. flavipes* and that of members of the millipede species *Narceus americanus* (Walker et al. 2009) and the cockroach species *Cryptocercus punctulatus* (Garrick et al. 2017). Found throughout the Appalachians, the nocturnal millipede genus *Narceus* resides in rotten logs during the daylight hours, feeding on decomposing leaf matter at night. A study which consisted of 269 individuals from 96 locations throughout the Appalachians was conducted to research glaciation effects on the species' distribution of genetic diversity. Mitochondrial DNA was analyzed together with paleoclimatic data to locate possible habitat refugia for *N. americanus* during the Last Glacial Maximum. The resulting genetic groups identified from the phylogenetic analyses showed distinct clusters in their geographic distribution

(Walker et al. 2009). As for *C. punctulatus,* these cockroaches feed on dead wood debris in the Southern Appalachian mountain region. The sample size for the study consisted of 95 individuals collected from 95 rotting logs located in seven states throughout the southern area of the Appalachians. Mitochondrial genes (COI and COII) were amplified to provide the data set for phylogenetic analyses. After building a phylogenetic tree with these DNA sequences, the species appeared to form five well-supported clusters, each of which were geographically cohesive (Garrick et al. 2017). As a group, the termite, millipede and cockroach species all showed phylogeographic structure within the same study region considered here.

One difference in the comparison of termites to the millipede and cockroach species is that termites are flight-capable, while the other two species are not. Termites are eusocial insects that have different castes, one of which is alates (i.e., winged reproductive individuals); during specific climatic conditions, mating flights occur resulting in partnering of male and female alates. Flight distances are relatively short ranging from a few meters to 1 kilometer (Vargo & Husseneder 2009). Sex pheromones emitting from the female assist the male in finding his mate. A study of *R. flavipes* showed that 80% of founding partners of a colony are composed of unrelated individuals (Vargo & Husseneder 2009). Conversely, *N. americanus* millipedes and *C. punctulatus* woodroaches are flightless, relying solely on walking to find mates. It is interesting to note that although these three species differ in their dispersal capabilities, they all seem to show strong phylogeographic structure. Although *R. flavipes* is a flight-capable species, the short distances they fly may nonetheless promote genetic differentiation over the

spatial scale of the study area, as is this case for flightless invertebrates such as millipedes and woodroaches.

Climatic cycles

Reasons for the phylogeographic structure seen in *R. flavipes* may, in some part, be due to the glacial/interglacial cycles during the Pleistocene. As mentioned earlier, forests retreated downslope during periods of colder climates, and those forests subsequently moved back upslope during warmer periods (Davis 1983). This cyclic fragmentation and reconnection of the forest habitats likely contributed to the genetic diversity seen within low-mobility, forest-dependent species. Invertebrate species such as termites depend on the decaying wood to survive, and so the historical changes in forest distribution may have affected genetic differentiation within the species (Davis 1983). Continued research in this field hopefully will reveal additional details about relationships between forest fragmentation and genetic diversity within species.

Limitations and Solutions

One limitation in the study was that only mtDNA was analyzed. The phylogenetic tree was constructed using the concatenated COI and COII genes, but given that all mtDNA genes are inherited as a single unit, collectively they represent only one genetic locus. Further research isolating multiple loci (i.e., including one or more nuclear genes) would help determine genetic groups within the focal termite species, and their phylogeographic structure. I did amplify and sequence a nuclear gene (nuclear endo-beta-1,4-glucanase) in my research, but these data were not used in the analyses presented

here. This nuclear gene showed genetic variation within species, which is helpful in determining genetic groups, and so it should be useful for future work.

There is a geographic space in the region around northern North Carolina and southern Virginia and Kentucky that is missing sample data (seen in Figure 5, Panel A). Obtaining more samples from that region is important in determining the spatial distribution of each of the four genetic groups identified here. The missing samples could provide more support for the phylogeographic structure, or alternatively, they may reveal added complexity not seen in the present study. Future research should include a complete range of samples throughout the region that is being researched.

The manner in which comparisons to the millipede and cockroach species were made here represents another limitation of this research. The information used to compare patterns of phylogeographic structure in each of the two flightless species to the termite *R. flavipes* (i.e., the focal species of the present work) was *qualitative*. A formal comparative analysis of the three species, addressing the extent to which they exhibit similar phylogeographic structures, would provide a useful study because it would allow us to distinguish between species-specific vs. habitat-specific responses to past climatic changes.

REFERENCES

Davis, M.B. (1983) Quaternary history of deciduous forests of eastern North America and Europe. Annals of the Missouri Botanical Garden, 70:550–563.

Delcourt, P.A., Delcourt, H.R. (1998) Paleoecological insights on conservation of biodiversity: a focus on species, ecosystems, and landscapes. Ecological Applications, 8:921-934.

Emerson, B.C., Hewitt, G.M. (2005) Phylogeography, Current Biology, 15: R367-R371.

Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3:294–299.

Garrick, R.C., Sabree, Z.L., Jahnes, B.C., Oliver, J.C. (2017) Strong spatial-genetic congruence between a wood-feeding cockroach and its bacterial endosymbiont, across a topographically complex landscape. Journal of Biogeography, 44:1500-1511.

Hyseni C, Garrick RC (in review) The role of distributional shifts in shaping genetic structure of the eastern subterranean termite in the southern Appalachian Mountains. Ecology and Evolution.

Oregon State University PRISM Climate Group (2011) PRISM Climate Group, Oregon State University, Northwest Alliance for Computational Science and Engineering (NACSE).

Park, Y.C., Maekawa, K., Matsumoto, T., Santoni, R., Choe, J.C. (2004) Molecular phylogeny and biogeography of the Korean woodroaches *Cryptocercus* spp. Molecular Phylogenetics and Evolution, 30:450–464.

Rissler, L.J., Smith, W.H. (2010) Mapping amphibian contact zones and phylogeographical break hotspots across the United States. Molecular Ecology, 19:5404-5416.

Saitou N. and Nei M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the

Entomological Society of America, 87:651–701.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution, 30:2725–2729.

Ulyshen, M.D. (2016) Wood decomposition as influenced by invertebrates. Biological Reviews, 91:70-85.

Vargo, E.L., Husseneder, C. (2009) Biology of subterranean termites: Insights from molecular studies of *Reticulitermes* and *Copotermes*. Annual Review of Entomology, 54:79-403.

Walker, M.J., Stockman, A.K., Marek, P.E., Bond, J.E. (2009) Pleistocene glacial refugia across the Appalachian Mountains and Coastal Plain in the millipede genus *Narceus*: Evidence from population genetic, phylogeographic, and paleoclimatic data. BMC Evolutionary Biology, 9:25.

Williams, J.W., Shuman, B.N., Webb III, T., Bartlein, P.J., Leduc, P.L. (2004) Late-Quaternary vegetation dynamics in North America: Scaling from taxa to biomes. Ecological Monographs, 74:309–334.