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Colony Breeding Structure of *Reticulitermes* (Isoptera: Rhinotermitidae) in Northwest Arkansas

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Abstract

Termites, as social insects, have a complicated life cycle in which the colony breeding structure, that is the number of and origin of reproductives in a colony, can vary in relation to age and environmental factors. In this study, we used genetic methods to characterize the breeding structure of three species of *Reticulitermes* from three sites in northwest Arkansas and compared two habitats: undeveloped, forested sites and developed, agricultural sites. We found 57.1% of *R. flavipes* (Kollar) in northwest Arkansas (n = 28) were simple families, 39.3% were extended families and 3.6% were mixed families. Similarly, for *R. hageni* Banks (n = 23), we found 58.3% simple families, 33.3% extended families, and 8.3% mixed families. All of the *R. virginicus* (Banks) samples (n = 5) were simple families. For *R. flavipes* and *R. hageni*, the percentage of extended families is intermediate to southeastern and northern USA populations, corresponding to the intermediate seasonality and climate in Arkansas. The level of inbreeding in Arkansas, estimated via F_{IT}, was relatively high and similar to northern populations of *Reticulitermes*. There were significantly more extended family colonies at the developed site compared to the two undeveloped sites which contained more simple family colonies. This difference may occur as a strategy to cope with sparse resources in urban environments or as a consequence of different abiotic factors.

Introduction

The complicated life style of termites prevents traditional research methods from adequately assessing aspects of their biology. Initially, a *Reticulitermes* (Isoptera; Rhinotermitidae) subterranean termite colony is founded by a monogamous pair of winged alates, forming a simple family. This pair can be replaced by secondary reproductive descended from the original founding pair, changing the breeding structure of the colony to an extended family (Vargo & 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 & Nishida, 2001; Deheer & 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 (Vargo 2000; Dronnet et al., 2004) and applied to *Reticulitermes* termites, primarily along the East Coast of the USA and Europe (Vargo & Husseneder, 2009).

In *R. flavipes* (Kollar) populations examined in the southeastern USA, there are generally 75% simple families, 20% extended families, and 2% mixed families (Vargo & Husseneder, 2009). In populations of *R. flavipes* further north in the USA (i.e. Virginia, Delaware, Massachusetts, Nebraska), there are more extended families (41.4 – 71.4%) and higher levels of inbreeding that correlate to latitude and seasonal climatic variables (Ab Majid et al., 2013; Vargo et al., 2013). Breeding structure of *R. hageni* Banks and *R. virginicus* (Banks) have been determined in North and South Carolina and are primarily simple families (>75%) with no mixed families (Vargo & Husseneder, 2009). However, in

R. virginicus this value may be elevated since this species undergoes asexual queen succession (AQS) (Vargo et al., 2012). In this system, female secondary reproductives are produced parthenogenetically, and therefore maintain the genetic structure of a simple family (Vargo et al., 2012). Microsatellite genotyping workers to determine the breeding structure of a colony often cannot differentiate between simple and extended families in AQS species.

Although many studies have investigated subterranean termites in either developed or undeveloped settings, few have compared aspects of termite biology between these two environments. In its invasive range in France, Dronnet et al. (2005) compared the genetic diversity of *R. flavipes* in urban and rural areas and found no differences. Similarly, family structure was not found to be different in urban compared to rural areas for *Reticulitermes* in the eastern USA (Vargo & Husseneder, 2009). In Nebraska, there were more simple families of *R. flavipes* in developed sites compared to undeveloped sites that had more mixed families (Ab Majid et al., 2013; Ab Majid et al., 2018).

In Arkansas, three species of *Reticulitermes* occur: *R. flavipes, R. hageni,* and *R. virginicus* (Austin et al., 2004). These subterranean termites, especially *R. flavipes,* are economically important structural pests that cost Americans approximately $11 billion annually for prevention, treatment, and repair of termite damage (Su, 2002). In this study, we investigate family structure for these three species of *Reticulitermes* from three sites in northwest Arkansas. We also determined if there is a difference in family structure between developed and undeveloped areas.

Materials and Methods

Samples

Termites were collected from three sites in northwest Arkansas, USA (Fig 1): Lake Wedington unit of the Ozark St. Francis National Forest (OSFNF) (abbreviated: Lake Wedington) (36.13128, -94.3946), Lee Creek area of OSFNF (abbreviated: Lee Creek) (35.76183, -94.2738), and the University of Arkansas Agricultural Research and Extension Center, Fayetteville, AR (abbreviated: UARK Farm) (36.09908, -94.1659). Sites were separated by 17.7 to 35.4 km from one another. All samples were collected between July 2014 and Mar. 2016. Each sample represents a colony and multiple individuals were collected per colony. In northwest Arkansas, the mean annual temperature is 14.3 °C ranging from -4.5 to 32.1 °C, and the average annual precipitation is 1180 mm (Fick & Hijans, 2017).

Lake Wedington was created in the 1930s through the Northwest Arkansas Land Use Project that converted misused or idle farm land into recreational and national forest areas. From this location, 21 samples were collected from decaying logs (Table 1). Lee Creek is located south of Devil's Den State Park, in OSFNF. It is primarily secondary growth forest that was also developed in the 1930s through efforts of the Civilian Conservation Corps (Smith, 1992). Twenty-one samples were collected at this location (Table 1). The UARK Farm 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 agricultural plots. Fourteen samples (Table 1) were collected from both decaying logs and Sentricon® Termite Colony Elimination System bait stations (Dow AgroSciences, Indianapolis, IN, USA) containing untreated pieces of pine wood. Fifty-six samples representing the three species and three localities were examined in this study (Table 1).

Table 1. Number of samples of each termite by location.

Site	*R. flavipes*	*R. hageni*	*R. virginicus*	Total
Lake Wedington	10	10	1	21
Lee Creek	9	9	3	21
UARK Farm	9	4	1	14
Totals	28	23	5	56

Land use per site was calculated in ArcGIS Desktop v.10.5.1 (Environmental Systems Research Institute (ESRI), Redlands, California, USA) using World_Imagery base maps (source: ESRI, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGA, AeroGRID, IGN, and the GIS User Community, last updated September 2018). A buffer circle with a radius of 500 m was drawn around each sample location. Within this area, different sections were manually drawn and scored as forest, water, road, agricultural, impervious surface, or building and the percentage of area was calculated for each (Fig 2 A-C). Each site was classified as "developed" or "undeveloped" according to the dominant (> 50%) land use within this radius. Areas that were dominated by heavily manipulated land uses (e.g., roads, impervious surface, buildings, or agriculture) were classified as developed, whereas areas dominated by forest were classified as undeveloped.
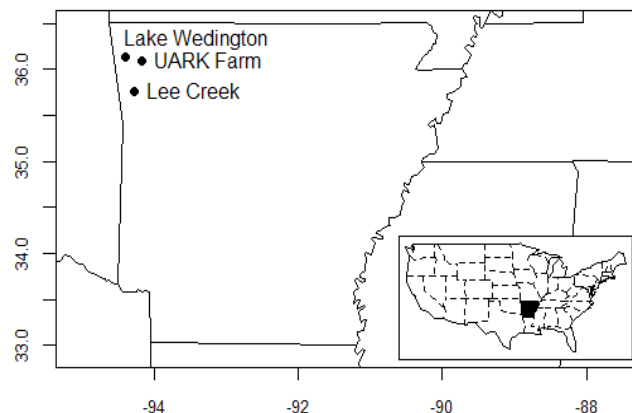

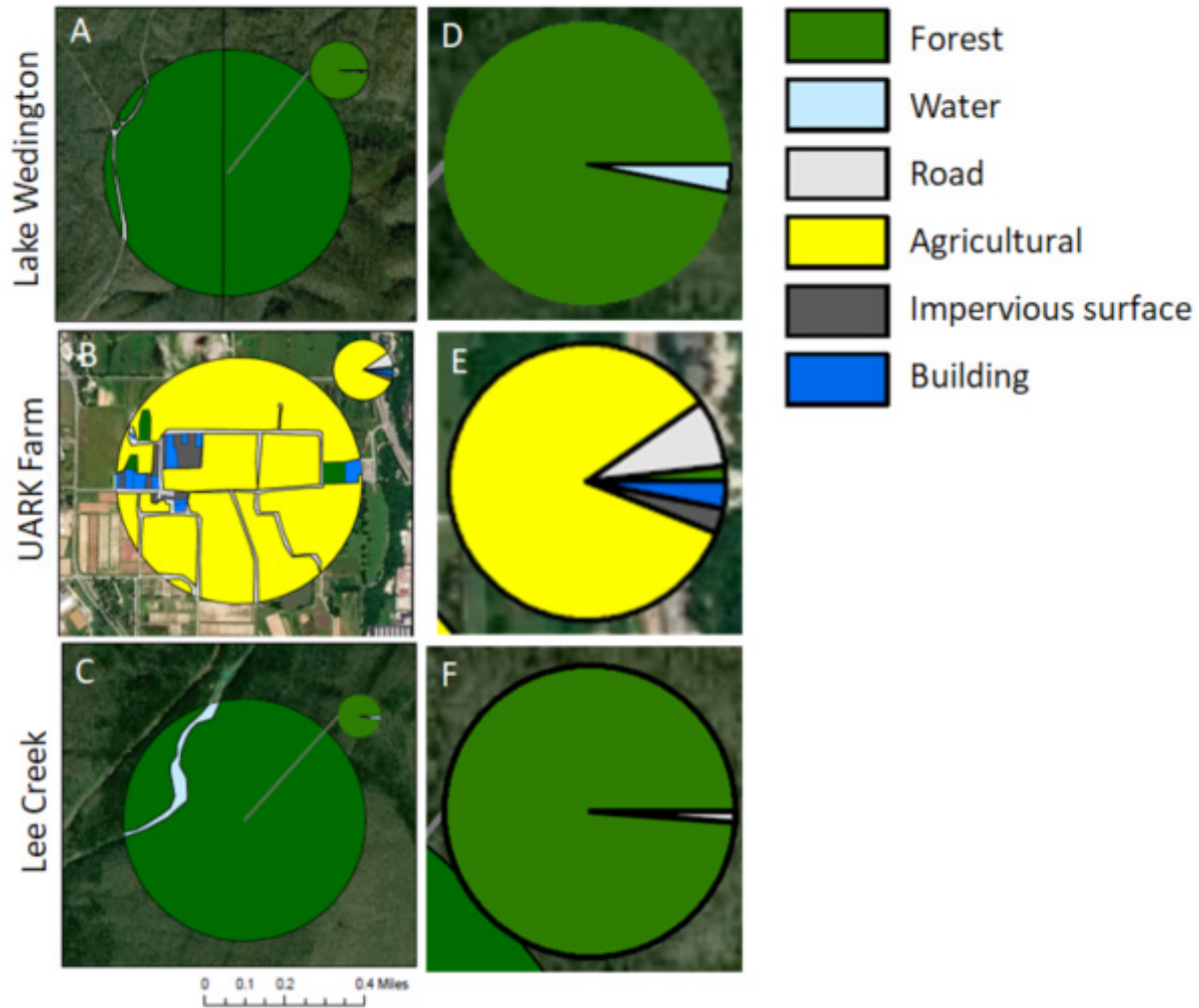Fig 1. Sampling sites in northwest Arkansas, USA.

Fig 2. Land use for a circle with a 500 m radius around each sampling site in northwest Arkansas (A-C) summarized as a pie chart for each site (D-F).

All samples were collected into 95% ethanol and voucher specimens are deposited in the Texas A&M University Insect Collection, College Station, TX, USA (#740). 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 the head from an individual specimen using a salting-out procedure with in-house reagents (Sambrook & Russell, 2001). The 16S gene region of mtDNA was amplified using the primers 16S: LR-J-13007 (5'-TTACGCTGTTATCCCTAA-3') (Kambhampati & Smith, 1995) and LR-N-13398 (5'-CGCCTGTTTATCAAA AACAT-3') (Simon et al., 1994). The PCR reagents followed Taylor et al. (1996) and the thermal cycler protocol consisted of 35 cycles of 94 °C for 45 s, 46 °C for 45 s, and 71 °C for 60s. Samples were purified and concentrated with VWR centrifugal devices (VWR, Radnor, PA) and sent to Eurofins Genomics (Huntsville, AL) for direct sequencing in both directions. 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), and GenBank.

Microsatellite Genotyping

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 & Russell, 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, USA). Each species had a unique PCR reaction mix that can be found in Supplementary Tables 1-3. The thermal cycler condition (modified from: Vargo, 2000) 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, USA) using an ABI Prism 3730 DNA Analyzer (Applied Biosystems, Life Technologies, Grand Island, NY, USA) or in house with an ABI 3500 Genetic Analyzer (Applied Biosystems, Life Technologies, Grand Island, NY, USA) with GeneScan 500 LIZ dye size standard (Applied Biosystems, Life Technologies, Grand Island, NY, USA).

Microsatellite Analysis

Alleles were scored with the microsatellite plugin for Geneious v6.1.8 (Kearse et al., 2012). Individuals and loci with more than 50% missing data were removed from analysis. To access if all samples represented unique colonies, genotypic differentiation between samples were calculated with Genepop (Raymond & Rousset, 1995). Following Vargo (2003), samples from unique colonies had pairwise genotypic differentiation values that were significantly (p < 0.05) different than zero, whereas samples of the same colony yielded non-significant p-values. For any repeatedly sampled colony, only the first sample obtained was used for further analysis.

Within each colony, breeding structure was determined. Alleles per locus were calculated and 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. Remaining colonies that were not classified as mixed families were divided into simple and extended families based on whether they had genotypes consistent with a monogamous pair of reproductive, and if so, whether the genotype frequencies differed from the expected Mendelian ratios (Vargo, 2003). The genetic variation partitioning among the individual (I), colony (C), and total population (T) were calculated in FSTAT v. 2.9.3 (Goudet, 2001) for each species separately. F_{IC} and F_{IT} estimate the level of inbreeding in individuals compared to others in the same colony and to others in the total population,

respectively (Vargo et al., 2013). F_{IC} was calculated for the extended families only because it is useful in estimating the number of reproductives present (Vargo & Husseneder, 2009). Differences in family structure by location were calculated with a Fisher exact probability test in R v. 3.4.1 (R Core Team 2013) because of the small sample size (i.e. more than 20% of cells are n < 5). Graphs were also generated in R v. 3.4.1 (R Core Team 2013).

Results

Species Identification

For *R. flavipes,* there were seven unique 16S mtDNA haplotypes from the 28 samples. For *R. hageni,* there were three unique 16S haplotypes in the 23 samples. And for *R. virginicus*, there were two 16S haplotypes in the 5 samples. Within a species, haplotypes were not exclusive to geographic location collected (Table 2).

Family Structure

A majority of pairwise genotypic differentiation values calculated for each species was significant, indicating that most samples represented unique colonies. To avoid sampling a single colony twice, eight samples with pairwise genotypic differentiation values close to zero were removed when comparing breeding structures between sites and species. Six of these samples were *R. flavipes* and two were *R. virginicus.* No samples of *R. hageni* were sampled twice. Combining all three species for the remaining samples (n=56), we observed 57.4% simple families, 37.7% extended families, and 4.9% mixed families (Fig 3, Table 3). All five colonies of *R. virginicus* collected were simple families. The F_{IC} values for extended family colonies of *R. flavipes* (0.055) and *R. hageni* (-0.082) were near zero, indicating that there were relatively high numbers of neotenics mating within the colony (~20; Thorne et al., 1999). The relatively high values of F_{IT} for all three species indicate these colonies are inbred (Table 3).

Family Structure by Site

While there was no significant difference in the frequencies of different family types between sites for each species alone: *R. flavipes* (Fisher exact probability test, p = 0.0638, n = 28), *R. hageni* (p = 0.2679, n = 23), *R. virginicus* (p = 1.00, n = 5); there is a significant difference in family structure type by site for all species combined (p = 0.012, n = 56). Generally, there were more extended families at the UARK Farm and more simple families at Lake Wedington and Lee Creek (Fig 3). There was no difference in family structure between Lake Wedington and Lee Creek for all three species combined (p = 0.1575). Interestingly, all mixed families for *R. flavipes* and *R. hageni* occurred at one site: Lake Wedington (Fig 3).

Table 2. 16S mtDNA haplotypes of each species by location.

16S Haplotype	Lake Wedington	Lee Creek	UARK Farm	Total	GenBank accession number
R. flavipes GG/SS	1	3	3	7	AY702099
R. flavipes J	0	0	1	1	AY441984
R. flavipes M	0	2	0	2	AY441987
R. flavipes MM	1	0	1	2	DQ001965
R. flavipes P	6	2	2	10	AY538741
R. flavipes R	0	1	0	1	AY603499
R. flavipes TT	2	1	2	5	DQ001972
R. hageni H1	7	8	2	17	AY257235
R. hageni H2	2	1	2	5	AY603505
R. hageni H4	1	0	0	1	EU484333
R. virginicus V1	1	2	0	3	AY257241
R. virginicus V15	0	1	1	2	EU259775

Table 3. Comparison of family structure types and inbreeding coefficients to other studies. Results from this study are shaded gray (modified from Vargo and Husseneder 2009, additional data from: Ab Majid et al. 2013, Perdereau et al. 2015, and Ab Majid et al. 2018).

Species/Population (N = no. of colonies)	Simple (Percent)	Extended		Mixed Families (Percent)	Overall F_{IT}
		Percent	F_{IC}		
Reticulitermes flavipes					
Central North Carolina (N = 319)	78.4%	19.7%	-0.209	1.9%	0.052
Charleston, South Carolina (N = 18)	72.2%	22.2%	-0.140	5.6%	0.030
Eastern Massachusetts (N = 22)	27.3%	59.1%	0.097	13.6%	0.289
Lincoln, Nebraska (N = 8)	28.6%	0%	NA*	71.4%	0.166
Nebraska (urban structures) (N = 20)	85.0%	0%	NA	15.0%	0.111
Central Tennessee (N = 48)	NR†	NR	0.260	NR	0.386
Northwest Arkansas (N = 28)	**57.1%**	**39.3%**	**0.055**	**3.6%**	**0.250**
New Orleans, Louisiana (N=20)	0%	50%	0.060	50%	0.362
Reticulitermes hageni					
Raleigh, North Carolina (N = 15)	86.7%	13.3%	-0.257	0%	0.357
Charleston, South Carolina (N = 21)	95.2%	4.8%	NA	0%	0.140
Northwest Arkansas (N = 23)	**58.3%**	**33.3%**	**-0.082**	**8.3%**	**0.394**
Reticulitermes virginicus					
Raleigh, North Carolina (N = 8)	75%	25%	-0.332	0%	0.037
Charleston, South Carolina (N = 4)	100%	0%	NA	0%	-0.04
Northwest Arkansas (N = 5)	**100.0%**	**0.0%**	**NA**	**0%**	**0.241**

*not applicable, †not reported

Discussion

Variation in breeding structure has been observed in different species and populations although the cause of this variation is unclear (Vargo & Husseneder, 2009). In *R. flavipes*, Vargo et al. (2013) found this variation in breeding structure and inbreeding was correlated to mean annual temperature and seasonality. In cool, moist habitats, there were higher levels of inbreeding and more extended families (Vargo et al., 2013). In Arkansas, our results align with this large-scale model but we also found differences in family structure between sites. This pattern of family structure aligns with the land use analysis where Lake Wedington and Lee Creek are undeveloped with mainly forest (>96%) whereas the UARK Farm is a developed site that has a wider variety of land use and is primarily agricultural land (>80%) (Fig 2 D-F). In undeveloped, forested sites, there are more simple families compared to developed sites that have more extended families.

Across these three species of *Reticulitermes* in northwest Arkansas, only 57.4% of the colonies sampled were simple families, 37.7% were extended families, and 4.9% (2 samples) were mixed families (Fig 2, Table 3). Compared to previous studies, the percent of *R. flavipes* extended families is intermediate between southeastern (low percentage) and northern USA populations (high percentage) (Table 3) which corresponds to the intermediate seasonality in Arkansas (Vargo & Husseneder, 2009; Vargo et al., 2013). For *R. hageni,* there are more extended families than was previously reported in North and South Carolina (Vargo & Husseneder, 2009). In *R. virginicus*, the samples from northwest Arkansas are similar in family structure to South Carolina (Table 3) (Vargo & Husseneder, 2009); although, this value may also be elevated because of the potential for asexual queen succession in this species (Vargo et al., 2012). Our methods may have been unable to distinguish between simple and extended families if parthenogenetic neotenics were present. For *R. flavipes,* the F_{IC} value for extended families is positive but close to zero and similar to what was observed in Louisiana and Massachusetts indicating many neotenics reproducing in the colony. The F_{IT} value estimated for *R. flavipes* in Arkansas is relatively high and similar to more populations in Tennessee and Massachusetts indicating a similarly high level of inbreeding. For *R. hageni* and *R. virginicus*, the F_{IT} values are closer to the North Carolina values, which for *R. virginicus* are opposite of the family structure percentages that were more similar to South Carolina.
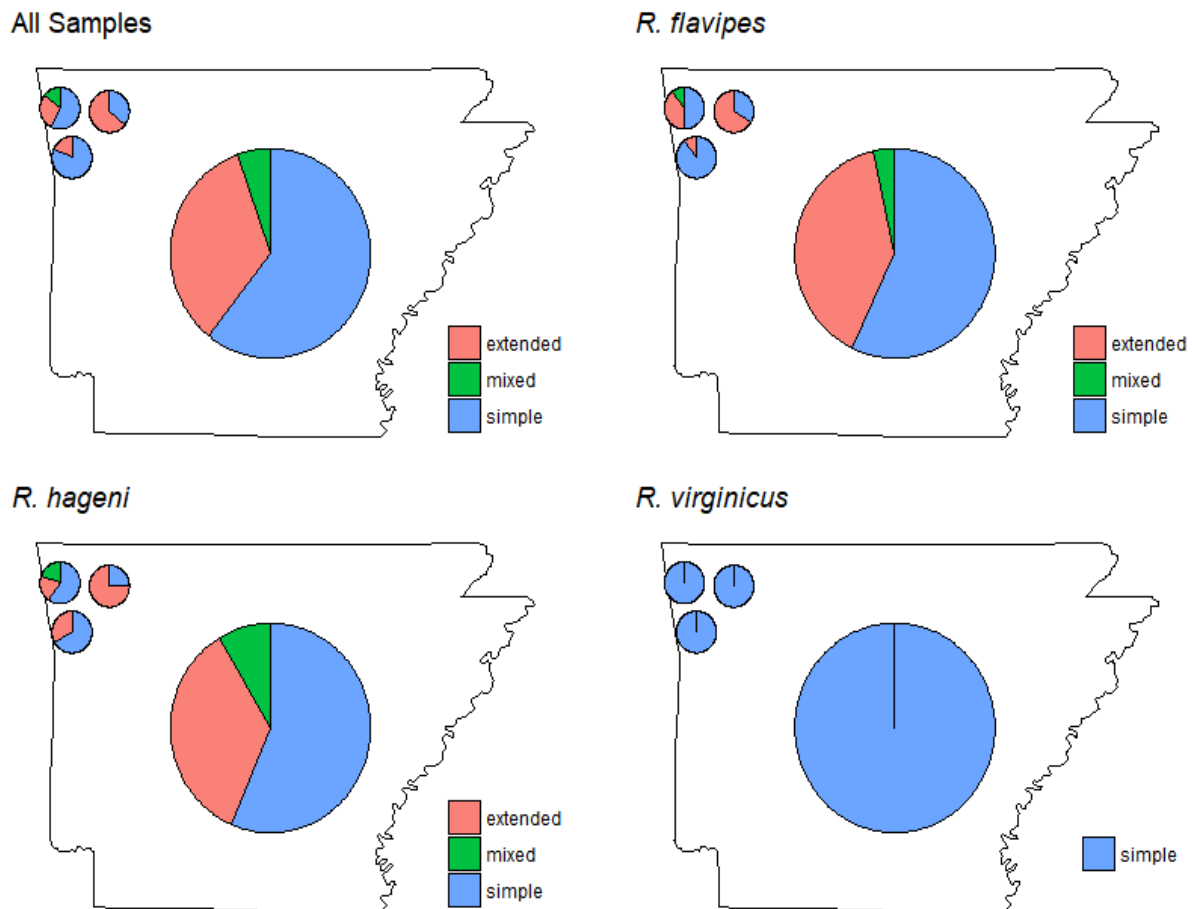
Fig 3. Family structure (simple, extended, or mixed family) comparison for the three different species (*R. flavipes, R. hageni,* and *R. virginicus*) and three different sampling locations in northwest Arkansas.

We found that the developed site, the UARK Farm, had relatively more extended family colonies of *R. flavipes* and *R. hageni* compared to the undeveloped sites (Fig 3) corresponding to differences in land use (Fig 2). This is the first report of this pattern of colonies of different family structure at developed vs. undeveloped sites. This stands in contrast to two studies in Nebraska on undeveloped (Ab Majid et al., 2013) and developed (Ab Majid et al., 2018) sites that found no extended families at either site but generally more simple families at developed sites compared to mixed families on undeveloped sites. However, this study of undeveloped sites in Nebraska represents a high outlier in the percent of mixed families (Table 3) and has a small sample size (n = 8).

As inferred from the genotypic differentiation, most samples represent unique colonies. There are several instances, however, where the same colony was sampled multiple times. Two samples collected from Sentricon® Termite Colony Elimination System bait stations within several meters of each other at the UARK Farm 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. Two samples from Lee Creek in fallen logs also

represent the same colony and were collected within several meters from each other. 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 to better understand how these species that appear to be inhabiting the same niche are able to coexist and partition space and resources.

Overall, we observed a relatively high percentage of extended families in northwest Arkansas. This is more than what has been observed in much of the eastern USA with the exception of northern populations. We further found a significant difference in family structure between our sampling sites that can be described by the land use with undeveloped, forested sites having more simple families and developed, agricultural sites having more extended families. This may reflect different strategies dependent on the abundance of resources. In the developed site there are less suitable resources to colonize compared to the undeveloped site, making inheriting the natal nest as an extended family potentially more profitable than attempting to found a new colony. Alternatively, this difference may be explained by adaptation to differences in temperature and moisture levels caused by urban heat islands or urban landscape management practices like irrigation, etc.

These results highlight the importance of understanding how termite biology shifts as they invade urban habitats.

Acknowledgements

Authors Contribution

MAJ, ALS, and ADT conceived and designed the project. MAJ collected the data. ADT, ELV, and MAJ conducted the data analysis. MAJ wrote the manuscript with support from ADT, ALS, and ELV.

Supplementary Material

http://periodicos.uefs.br/index.php/sociobiology/rt/suppFiles/4376/0
http://dx.doi.org/10.13102/sociobiology.v66i3.4376.s2350

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