Effect of Different Climate Environment on Foraging Behavior, Colony Genetic Organization, Movement and Breeding Pattern of Subterranean Termites (*Reticulitermes flavipes*)

by

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Abstract

Limited data exist on agonistic or aggressive behavior of subterranean termites of the same species but from different colonies. We conducted research to determine if subterranean termites, *Reticulitermes flavipes* (Kollar) from different colonies may express different foraging, agonistic and feeding behavior during colony fusion at different temperatures. Fifty *R. flavipes* workers from two different colonies (colony #1, undyed and colony #2, dyed with Nile blue-A) were placed in separate petri dishes (feeding chambers) and connected with polyethylene tubes to a common petri dish (feeding chamber) for foraging interaction at 15, 20, 23, 25 and 30°C. Each treatment had three replications. Data were recorded on foraging, agonistic or aggressive behavior and food consumption of termite workers at 1, 3, 24, 72 h and 7, 14 and 21 d intervals. Our data showed that both colony members did not display agonistic behavior and fused together at 15-25°C with maximum food consumption at 20°C. Food consumption was not significantly different ($P \geq 0.05$) at 15-30°C but the 30°C was not the optimum temperature for termite survival. Microsatellite genotyping data from the field indicated eight termite's colonies have variable breeding patterns over the three years termites sample collection from the same colonies. Overall breeding pattern of the eight colonies changed from simple to mixed family colonies throughout the three years and back to simple family colonies type during season transition from fall to winter in 2009, 2010 and 2011. F-statistics and relatedness analysis showed that the colony founded by unrelated pair of reproductive with high relatedness value ($r > 0.85$). Our studies indicated that termite colonies could form via the fusion of over the time period under natural conditions and probably influence by seasonal temperature change. Therefore, this research will lead us to better IPM approach strategies to control termite infestation effectively by understanding termite strategies forage for food under different climate environment at the molecular level.

Key words: Molecular ecology, population genetics

Introduction

Termites foraging behavior includes strategies of chemical communication while searching for food (Reinhard et al., 1997). Termite foraging in the soil also requires acceptable moisture and moderate temperatures (Campora and Grace, 2001). Food resources when located are analyzed (Lys and Leuthhold, 1991; Campora and Grace, 2001). If the food is acquired and consumed, the foragers lay a pheromone trail back to the nest. A primary gallery is constructed around this recruitment trail (Lys and Leuthhold, 1991; Reinhard et al., 1997). Exploration for a food source becomes the new center of activity after the current food is consumed and the probability of searching the same area is. Furthermore, environmental climate and temperature play an important role in termite foraging searching for food (Reinhard et al., 1997; Campora and Grace, 2001).

In addition, it is important to screen a large number of colony sites in natural conditions to determine the founders of termite colonies. Both the primary and secondary reproductives are usually difficult to collect; however, the genetic structure of colonies is usually inferred by genotyping groups of workers (Vargo 2003a, b; DeHeer and Vargo, 2004; Perdereau et al., 2010; Vargo and Hussender, 2009). New molecular genetic techniques are providing exceptional new insights into the biology of subterranean termites. In addition to elucidating such basic processes as development and caste differentiation, molecular techniques give us a window into the breeding structure, as well as colony and population dynamics that has remained elusive owing to the cryptic nesting and foraging habits of termites (Vargo and Hussender, 2009). DeHeer and Vargo (2004) showed that two independent mature colonies were fused in a single colony and represented as a typical mixed family during three successive field seasons. By understand the breeding patter

The first objectives of the study was to track the breeding structure pattern and colony genetic organization searching for food at natural forested area in Nebraska USA at different seasonal period for three year. The second objective was to observed different temperature setting under control environment condition that effecting termite tunneling activity forage for food in the laboratory.

Materials and methods

Field collection Termite workers from each of 8 different feeding sites within forested sections of Wilderness Park on the outskirts of Lincoln, Nebraska, United States were collected. The samples were collected from the exact same sites from May 2009, May 2010, November 2010 and May 2011. From each log (or feeding site), we collected a minimum of 10 termites and recorded the geographic coordinates using a hand-held GPS unit (SporTrak™ Map, Thales Navigation, Santa Clara, CA). Immediately following collection, all workers from each collection log were preserved in 95% ethanol and stored at -20^{o} C until DNA extraction.

DNA extraction Genomic DNA was extracted from each of 10 worker heads from each feeding site using a Qiagen DNeasy Kit (QIAGEN, USA). The manufacturer's protocols were followed except that treatments with Proteinase K solution and RNase were omitted and DNA was eluted in 80 µl of 1X TE solution. The concentration of DNA in each extract was quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Inc. Wilmington, DE, U.S.A).

Microsatellite genotyping Each termite worker was genotyped at seven microsatellite loci: *Rf 1 - 3, Rf 5 - 10, Rf 6 – 1, Rf 11 – 1, Rf 1 – 2, Rf 15 - 2* and *Rf 21 – 1* (Vargo 2000). For each microsatellite marker, the forward primer was labeled with one of three WellRED Fluorescent labels (D2, D3, and D4) for running on the Beckman CEQ 8000 (SIGMA-Proligo, The Woodlands, Texas). The PCR reactions were set up in 96 well plates in 15 µl reaction mixtures containing 10X PCR buffer, 50 mM $MgCl_2$, 10 mM Dntp mix, 0.025 µM forward primer 0.025 µM reverse primer, 100 units Taq DNA polymerase (Invitrogen) 2.0 ng DNA template. All loci were amplified using a PCR thermal cycler program with an initial denaturation step of 95˚C (30s), followed by 35 cycles at 95˚C (30s), 54˚C (30s), and 72˚C (30s). The reaction was terminated with one cycle of 72˚C (5 min) and then held at 4˚C until removed from the PCR thermal cycler. Fragments were separated and sized by capillary electrophoresis using a Beckman CEQ 8000 Genetic Analyzer in conjunction with 400 bp size standard.

Colony identity Allelic diversity, expected and observed heterozygosity were calculated using Fstat 2.9.3.2 (Goudet , 2001). Exact tests of genotypic differentiation were performed using GENEPOP on the Web (Goudet et al., 1996) http://wbiomed.curtin.edu.au/genepop/index.html) to determine if termites from different collection points belonged to the same colony or not. When two independent samples of workers are drawn from the same colony, we are sampling from the distribution of genotypes within that colony. Conversely, when two samples of workers are drawn from two different colonies, we are sampling from two different distributions of genotypes. This is true, regardless of the specific breeding structure of colonies involved. Therefore, if we test for differences in genotype frequencies between 2 samples of workers, we expect the test to be significant if they come from different colonies and non-significant if they come from the same colony.

Colony breeding structure Breeding structure was classified using the techniques of Vargo (2003a) and DeHeer and Vargo (2004). Individuals from the same colony were grouped together to determine the simplest breeding system that could be invoked to explain the genotype distributions within each colony. If colonies consisted of workers whose genotypes could be reconstructed by assuming a single mother and father, the frequencies of the observed genotypes did not differ significantly from those expected under simple Mendelian patterns of inheritance for this hypothetical pair (using a G-test summed over all loci, e.g., Vargo, 2003b), then the colony was classified as a simple-family colony, headed by the original colony-founding pair of reproductives. Colonies that had five or more alleles at least one locus could be unambiguously identified as mixed colonies headed by more than one pair of primary reproductives. In the case of colonies that do not fit the expected genotype frequencies for progeny of a simple family that had four or few alleles at all loci, the breeding structure could not be resolved unambiguously.

Colony genetic structure and relatedness co-efficiency To gain additional insight into the genetic structure of the colonies, F-statistics and relatedness coefficients were computed using the program FSTAT v. 2.9.3.2 (Goudet 2001). F-statistics followed the notation of Thorne et al. (1999), with the subscripts I, C and T representing the individual, colony, and total components of genetic variation, respectively. The 95% confidence intervals were obtained by bootstrapping over loci 10,000 times, and the significance of the coefficients was tested by permuting alleles among individuals. The overall inbreeding coefficient (F_{IT}) reflects the deficiency of heterozygotes because of non- random mating within the total samples in eight sampling locations. F_{CT} estimates the amount of genetic differentiation (allele frequency differences) among colonies. F_{IC} is a colony-level inbreeding coefficient which is perhaps the most useful measure as it varies with the number of reproductives as well as their spatial distribution within colonies. F_{IC} provides information on the number of reproductives and their relatedness. It is expected to be negative in simple families headed by a pair of reproductive (Thorne et al., 1999; Copren, 2007).

Temperature Setting Percival growth chambers (Percival Scientific Inc., Perry, IA) were preset at 15, 20, 23, 25 and 30^oC. Twenty termite workers plus one soldier were placed in a plastic petri dish (100 by 15 mm) with moist sand and corrugated cardboard. Later, these petri dishes with termites were placed in growth chambers at preset temperatures to ascertain that termites could survive for 30 d.

Feeding Arenas Feeding arenas were similar to those used by Binder (1988). Each arena consisted a set of three polyethylene petri dished (feeding chambers) (100 mm diameter x 15 mm height, BD Falcon company, Franklin Lakes, NJ) and connected with 2 cm long pieces of 6.35 mm diameter polyethylene tubing to accommodate approximately 1,000 termites (Fig. 1). Each arena consists of ~5 g sterile sand. The pinewood pieces (1.0 cm length x 1.0 cm width x 2.5 cm height) were oven dried overnight (80^oC), cooled and weighed before their use. Each pair of pinewood piece weighed ~1.5 g. One of the termite colony groups was stained with 0.1% (wt/wt) Nile Blue A by a no choice feeding of stained filter paper (Whatman No. 1, 9.0 cm in diameter) for 5-7 d (Abdul Hafiz et al., 2007).

Feeding and Food Consumption Fifty undyed termite workers from colony #1 were placed in the petri dish (feeding chamber) proximal of feeding arena and 50 Nile blue-A dyed termite workers from a colony #2 were placed in the petri dish (feeding chamber) distal to the feeding arena, leaving the middle petri dish (feeding chamber) unoccupied (Fig. 1). Termites foraging activities were observed at intervals of 1, 3, 24, and 72 h and 7, 14 and 21 d. Food consumption was recorded at the end of 3 wk after dismantling the pairing feeding arenas. The pair of pinewood pieces at each arena were cleaned, oven dried overnight (80^{o}C), cooled and weighted. The experiment design was the completely randomized block and each treatment had three replications. Analysis of variance (ANOVA) and *t*-tests (LSD) were conducted using SAS (SAS Institute Inc., 2000, Carey, NC, USA) to test for significant differences in feeding ($P \geq 0.05$).

Colony Fusion Fifty undyed termite workers from colony #1 were placed in the petri dish (feeding chamber) proximal of feeding arena and 50 Nile blue-A dyed termite workers from a colony #2 were placed in the petri dish (feeding chamber) distal to the feeding arena, leaving the middle petri dish unoccupied (Fig. 1). The arenas were left on the laboratory bench for 30 min for acclimation. Each petri dish in the feeding arena was labeled reflecting appropriate treatment and the replication. The arenas were placed in 60.96 x 41.99 x 14.94 cm hard plastic boxes (Bella, Leominster, MA) covered with aluminum foil. Finally, the plastic boxes were placed in growth chambers that were programed for 15, 20, 23, 25 and 30^{o}C. The sand in each petri dish was moistened weekly or as needed. Inter-colony pairings were monitored at intervals of 1, 3, 24, 72 h and 7, 14 and 21 d. After 3 wk of incubation, each feeding arena was dismantled and the numbers of undyed and blue dyed termites in each of the three petri dishes were recorded. For each test arena, connecting tubes were considered as part of the respective petri dish (feeding chamber). All colony pairing were categorized according to Fisher et al. (2004) as (a) sharing both nesting space food resources, (b) sharing food resources but maintaining separate nesting material or (c) maintaining separate nesting space not sharing food resources. Colony fusion was defined as the sharing of both nest sites and food resources (Fisher et al., 2004).

Results and discussion

Breeding pattern In May 2009, all 8 colonies were classified as simple family colonies (Table 1). In May 2010, most of the colonies (62.5%) had converted into mixed family colonies. Colonies number one, three, four, five, six and seven converted into mixed colonies. Meanwhile, colony number three converted into an unknown colony. The remaining colony number two and eight remains as simple family colony type (Table 1). However, during the transition of the season from fall to winter in early November 2010, all eight colonies were converted into simple family colonies (Table 1). Moreover, samples from the same eight colonies in May 2011 showed that the breeding structures were changed again mostly to mix family colony type (62.5%). Colony number one was converted into an unknown family type; meanwhile colony number two, three, five, seven and eight had converted into mixed colony type. Only colonies number four and five remained as simple family colonies (Table 1). *R. flavipes* populations spanning much of the eastern seaboard of the United States show strong variation in colony breeding structure, with a greater proportion of mixed colonies and higher levels of inbreeding in northern populations (Vargo and Carlson, 2006; DeHeer and Kamble, 2008). The simple families identified from colonies 1-8 in May 2009 were converted into mixed family colonies in May 2010. This is the first time that a transition from simple to mixed families has been observed in Nebraska with extreme cold weather during winter season. Transition from simple to extended family colony and mixed family colony is a part of the natural life cycle of subterranean termite colonies (Husseneder et al., 1999; Jenkins et al., 1999; Clement et al., 2001; Goodisman and Crozier, 2002). However, in November 2010, all eight colonies had changed back to simple family colonies. All F statistics values supported the conclusion of simple family colonies. Therefore, the change to simple family colony may be due to selective pressure of the extreme cold temperatures during late fall to early winter in November in Nebraska. In May 2011, all 8 colonies showed a transition mostly to mixed colonies and one unclassified family type. The three years' data on colony and breeding organization revealed that most of these colonies are likely to disperse in summer (May-June). Since simple family colonies in this study were not inbred, it can be concluded that most colonies were headed by the original founding pair of primary reproductive (Atkinson and Adams 1997, Husseneder et al. 1999, Jenkins et al. 1999)

Colony genetic structure and relatedness A negative F_{IC} value in May 2009 (F_{IC} = -0.661) for all 8 colonies indicated the presence of excessive heterozygotes and with high relatedness values (r = 0.823), thus they were referred to as the founders of the colonies which initiated by outbreed parents (Table 2). In addition all eight colonies represent presented levels of genetic differentiation among the colonies F_{CT} (0.441). In May 2010, the overall measure of inbreeding, F_{IT} was significantly greater than zero (Table 2) indicating a general deficit of heterozygosity compared to the expectations under Hardy-Weinberg genotypic equilibrium. The colonies that were classified as simple families had a significant negative F_{IC} (-0.296) indicating an excess of heterozygotes compared to a panmictic population with the same allele frequencies. This is consistent with the expected value of F_{IC} for a simple family (-0.209 to -0.33) (Vargo and Husseneder 2009). Furthermore, simple family colonies were genetically different with positive F_{CT} value (F_{CT} = 0.195) (Table 2). Mixed family colonies also had a negative F_{IC} (-0.106) indicating excessive heterozygosity similar to simple family colonies. Average relatedness values within simple family and mixed colonies were similar (Table 2). A high level of genetic differentiation existed among the colonies classified as mixed family colonies (F_{CT} = 0.245) (Table 2). In early November 2010, all the colonies changed back into simple family colony type with negative F_{IC} (F_{IC} = -0.8580). All of those 8 colonies had excessive heterozygosity similar to May 2009 with high relatedness values (r = 0.797) (Table 2). In addition F_{CT} estimates were significantly greater than zero in all 8 colonies, as shown by the permutation test ($P < 0.005$) showing significant genetic differentiation among colonies. In May 2011, workers from different colonies showed lower relatedness (r = 0.259) than previous years, suggesting the individual worker in the colonies were no longer close siblings with negative F_{IC} value (F_{IC} = -0.221). Low negative F_{IC} values (F_{IC} = -0.282) in simple family colonies indicated an excess of heterozygosity than the previous year. However, two of these simple family colonies were moderately genetically different with positive F_{CT} value (F_{CT} = 0.184), which lower than in previous years. Mixed family colonies also show medium excess heterozygosity with negative F_{IC} value (F_{IC} = -0.199) with moderate genetic differentiation among colonies (F_{CT} = 0.121) (Table 2). The low relatedness values in May 2011 suggested that positive and assortive mating might occur. The proportion of mixed colonies increased from year one (2009) to year two (2010). The shift from simple family colonies to mixed family colonies could be attributed to the early summer season in May where typically the alates go out to mate. In subterranean termites, mixed family colonies have been shown in only three species, *R. flavipes, R. grassei* and *R. speratus* where it involved the colony fusion mechanism (DeHeer and Vargo, 2004; 2008, Hayashi et al., 2007). In addition, the stability of mixed family colonies over the long term is unclear. The field data (DeHeer and Vargo, 2008) and lab (Fisher et al., 2004) indicated that the presence of multiple unrelated groups of 7reproductive in fused colonies for *R. flavpies* is generally short lived. In this study, a very high level of genetic differentiation occurred among all eight colonies over the three year period. During the dispersal of the alates, the dispersal and sex biased alate production are indirect mechanisms influencing the likelihood that colony founders meet in swarms (Husseneder et al., 1999). However, the role of kin recognition in partner selection in termites has not been widely studied despite the obvious consequences of this behavior for colony genetic structure and kin selection in general (Husseneder et al., 1999; Thorne et al., 1999). Intra- and interspecific patterns of variation in breeding systems over three years varied from the same collection point.

Food consumption, colony fusion and termite mortality Termites fed actively at 15-25°C and the highest feeding was at 20°C with mean wood consumption of 0.34 g and the lowest feeding consumption at 15°C. The 25° C was considered the upper limit with mean wood consumption of 0.29 g. However, the *t*-test (LSD) indicated no significant differences in wood consumption amongst all temperatures (15, 20, 23, 25 and 30°C) ($P \geq 0.05$). All fifteen termite colony-pairings (100%) shared both nesting areas and food resources at all temperatures until 21 d. In all colony pairings during the first hour at all temperature settings, the termites were still foraging within the original feeding chamber and searching for suitable nesting and food. At 25°C, all the pairings shared both nesting space and food resources. At 15, 20 and 23°C, only 33.3% of colony pairings shared both nesting space and food resources, while 66.7% termites shared food resources but maintaining separate nesting material. After 3 h, all the colony pairings were sharing both nesting spaces and food resources at 15-25°C. However, at 30°C only 33.3% of termites shared both nesting and food resources, while 66.7% termites shared food resources but maintained separate nesting materials. After 24 h, all the colony pairings at all temperatures shared both nesting and food resources, and the termites were very active in all feeding chambers. After 14 d, all the pairings still shared both nesting space and food resources except at 30°C. In addition, at 30°C the termites were not actively moving in colony pairing. After 21 d, the termites in colony pairings shared both nesting areas and food resources at all temperatures. Furthermore, termites in colony pairings were sluggish at 30°C after 21 d. Termites in colonies that fused/merged were observed grooming, participating in trophallaxis and foraging with unrelated nest mates. Matsuura and Nishida (2001) reported that the fusion occurred in colonies with numerous nymphs preparing to molt into alates and they are more likely to be accepted in foreign colonies in lab conditions and so these observations may not be applicable to field colonies. However, DeHeer and Vargo (2008) showed that individuals originating from different families had identical mtDNA, but were unrelated at nuclear microsatellite loci. According to them, the data suggested some maternal inherited factors underlying colony fusion, but the nature of this mechanism is unknown (DeHeer and Vargo, 2008). According to our data, the field collected subterranean termite colonies from different locations can merge/fuse together in the laboratory conditions at 15-25°C. However, the temperature >30°C may delay the termite colony-fusion process and had 65-75% mortality. The optimum temperature for the pairing-termites was 25°C with only 15-41% mortality. In addition, at 15, 20 and 23°C termites had <50% mortality. Based on *t*- test analysis, termite mortality did not differ significantly at 15 to 25°C except for 30°C ($P \leq 0.05$). Temperature setting from 15 to 25°C also can be ideal temperatures for the termite pairings and colony fusion. Overall, temperatures have a significant effect on termite mortality ($P \leq 0.05$).

Conclusion

The field data indicated that seasonal change can influence the breeding and population genetics pattern thus influence its foraging behavior. Meanwhile in the laboratory bioassay, temperatures around 15-25° C are considered to be optimal for the termites to be actively interacting with each other. In addition, this study showed that the colony genetic and spatial organization in *Reticulitermes* termites seems to be variable throughout the three year season. We anticipate that the results of the study will yield a fuller understanding of the forces underlying colony fusion and foraging behavior in subterranean termites caused by temperature (weather) changes. Additionally, information from this study can lead us for better termite management.

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Table 1. Variability of microsatellite loci and basic summary statistics for 8 colonies of *Reticulitermes flavipes* collected from Wilderness Park, Lincoln, Nebraska (2009-2011)

Colony Summer 2009	Family Structure	No of alleles detected per locus/per sample							
		Rf 11-3	Rf 5-10	Rf 6-1	Rf 11-1	Rf 11-2	Rf 15-2	Rf 21-1	Mean number of alleles
1	Simple	2	2	2	3	3	1	2	2.14
2	Simple	2	2	2	3	1	3	3	2.28
3	Simple	1	2	2	2	3	2	2	2.00
4	Simple	1	2	2	2	2	3	2	2.00
5	Simple	3	2	3	3	2	2	3	2.57
6	Simple	2	2	2	1	3	2	2	2.00
7	Simple	2	1	3	2	2	1	2	1.86
8	Simple	1	1	2	1	2	2	3	1.7
Summer 2010									
1	Mixed	4	2	2	4	5	4	5	3.7
2	Simple	2	1	2	3	4	2	4	2.5
3	Unclassified	3	4	2	2	4	4	4	3.28
4	Mixed	4	2	2	5	4	4	2	3.28
5	Mixed	3	5	2	2	6	2	4	3.43
6	Mixed	4	3	2	1	4	5	4	3.29
7	Mixed	3	3	2	3	4	5	3	3.28
Fall/Winter 2010									
8	Simple	3	4	2	2	2	2	3	2.57
1	Simple	2	2	2	2	2	2	2	2.00
2	Simple	1	2	2	3	1	2	2	1.86
3	Simple	3	2	2	2	2	2	2	2.14
4	Simple	2	2	2	2	1	2	2	1.86
5	Simple	2	2	2	1	2	2	2	1.86

6	Simple	3	2	2	2	2	3	3	2.43
7	Simple	2	2	2	3	2	3	2	2.29
8	Simple	1	2	2	2	2	2	2	1.86
Summer 2011									
1	Unclassified	4	2	2	4	4	2	4	3.14
2	Mixed	2	3	5	3	7	3	3	3.71
3	Mixed	3	2	2	3	4	6	2	3.14
4	Simple	2	2	3	4	4	4	3	3.14
5	Mixed	2	2	2	3	3	2	5	2.71
6	Simple	3	2	2	3	3	2	4	2.71
7	Mixed	1	2	2	3	3	4	5	2.85
8	Mixed	2	2	2	3	3	3	7	3.14