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Colony Genetic Organization and Breeding Pattern of Subterranean Termite (*Reticulitermes flavipes*) over the Three Field Seasons in Nebraska, U.S.A

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Abstract

In this study, we repeated sampling from individual eight colonies from Wilderness Park, Lincoln, Nebraska separated by at least 200 meters over a three years period and during seasonal change (fall-winter) from 2009 to 2011. Ten workers from each site were genotyped at seven microsatellite loci. The results show that 62.5% of simple family colonies converted into mixed family after a year. The 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 the transition from fall to winter in 2010. F-statistics and relatedness analysis showed that the colony founded by unrelated pair of reproductives have high relatedness value (r>0.85) within colony. Our data indicated that termite colonies could form through the colonies fusion over time under natural conditions and probably could be influenced by seasonal temperature change.

Keywords: Breeding system; Colony fusion; Colony genetic organization; *Reticulitermes flavipes*; Microsatellites

Introduction

Termites foraging behavior includes strategies of chemical communication while searching for food [1]. Termite foraging in the soil also requires acceptable moisture and moderate temperatures [2]. Food resources when located are analyzed [2,3]. 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 [1,3]. 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 fulfilled [1,2].

In addition, worker foraging strategies in searching for food allow formation of complex family structures when workers from different colonies forage at the same locations, especially when different colonies share the same tunnel systems, or when mature colonies fuse [4]. All of these mechanisms can explain the scattered appearance of complex family structure in several *Reticulitermes* spp. [5,6], including *R. flavipes* [7-9].

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 [10-14]. 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 [14]. [12] showed that two independent mature colonies were fused in a single colony and represented as a typical mixed family during three successive field seasons.

The primary goal of the current study was to track the breeding structure pattern and colony genetic organization of a large number of *R. flavipes* colonies from natural forested areas in the Midwestern regions of the U.S.A over the course of three years from 2009 to 2011.

Materials and Methods

Field collection

We collected the termite workers from each of 8 different feeding sites within forested sections of Wilderness Park on the outskirts of Lincoln, NE (Figure 1). The distance between each different feeding sites were within the range of 500 m-800 m. The sites were selected based on termite active feeding on the wooden log. 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°C until DNA extraction.

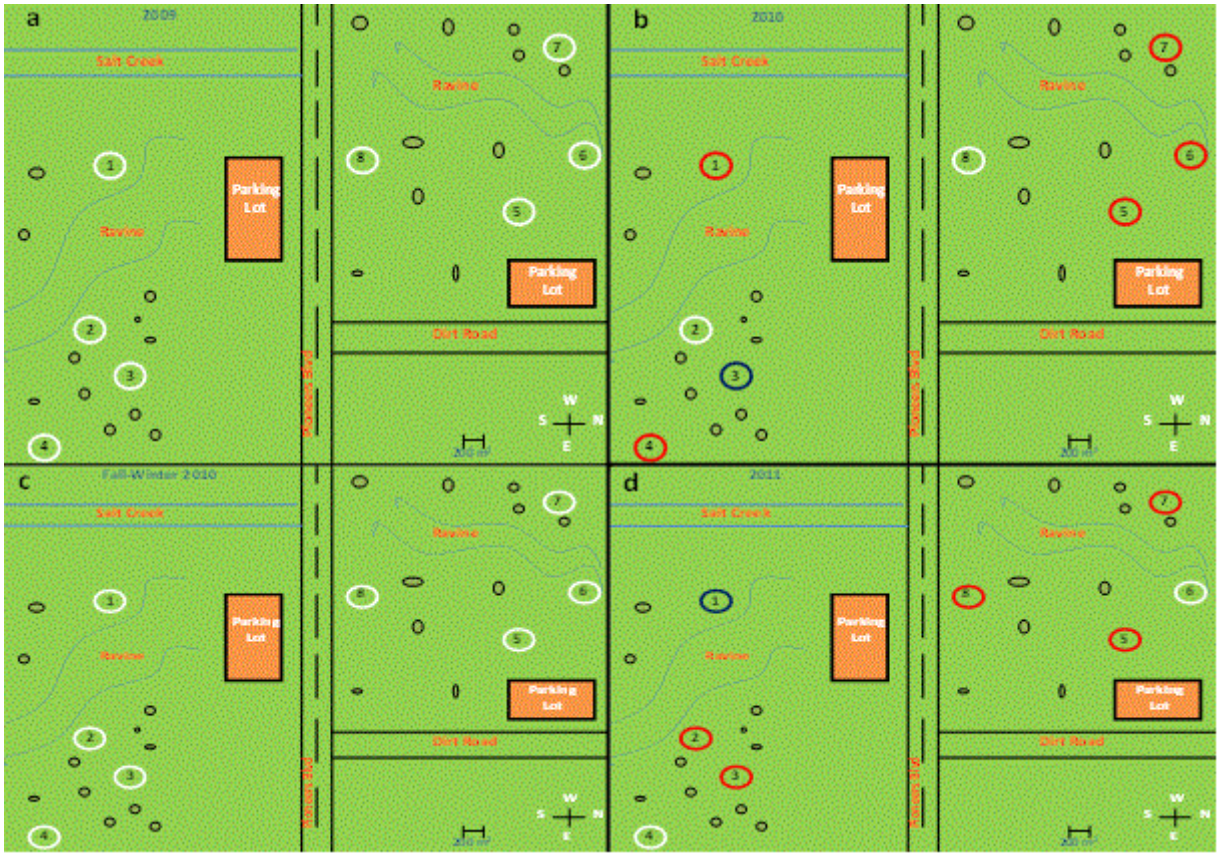

Figure 1: Location of termite colonies and breeding patterns over the three years season (2009-2011). White circles represent simple family colonies, red circles represent mixed family colonies and blue circle represent unclassified colonies. (a) Summer 2009 breeding pattern. (b) Summer 2010 breeding pattern. (c) Fall 2010 breeding pattern. (d) Summer 2011 breeding pattern.

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. 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. Data were analyzed and hand-scored using CEQ 8000 Fragment Analysis Software version 8.0, and a subset of samples for each locus was confirmed by a second human reader.

Colony identity

Termites collected from 8 sites were micro-morphologically identified as *R. flavipes* according to [15]. Allelic diversity, expected and observed heterozygosity were calculated using Fstat 2.9.3.2 [16]. Exact tests of genotypic differentiation were performed using GENEPOP on the Web 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 consist of minimum of ten individuals 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 [10,12]. 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., [11]), 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. This is because it is not possible to distinguish between an extended family colony that contains secondary reproductives which is a mixed colony where the king and queen share the same four or fewer alleles.

Colony genetic structure and relatedness coefficiency

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 [17], 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 (FIT) reflects the deficiency of heterozygotes because of nonrandom mating within the total samples in eight sampling locations. FCT estimates the amount of genetic differentiation (allele frequency differences) among colonies. FIC 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. FIC 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 [17-21]. For simple families, FIC is expected to be strongly negative FIC, values should approach zero with increasing number of reproductives within colonies, and to become positive if there is positive assortive mating among multiple reproductive within colonies or if there is mixing of individuals from different colonies [13,17,22-25] Genetic relatedness among workers was estimated for each colony and averaged over colonies of the same site. The standard errors of the means were obtained by jackknifing over colonies. For the allelic frequencies and the average relatedness estimates, colonies were weighed equally.

Results

Colony identification and differentiation

Termites collected from 8 sites were micro-morphologicaly identified as R. flavipes according to [15]. The distance between collection points within colonies was up to 200-500 m apart. There was strong highly significant differentiation among the *R. flavipes* among the pairwise sample points ($P<0.0001$) in 2008 untill 2011. Therefore, all the eight sample points represented different colonies throughout three consecutive years (2009-2011).

Breeding pattern

In May 2009, all 8 colonies were classified as simple family colonies (Table 1 and Figure 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 and Figure 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 and Figure 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 and Figure 1).

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

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 Genetic Structure and Relatedness

Colony Location (Wilderness Park, Lincoln, NE)	FCT	P-value	FIC	P-value	R
Empirical values					
Simple family colonies 2009 (n=8)	0.441	0	-0.661	0	0.823
	(0.267-0.571)		(-0.771--0.549)		(0.738-0.873)
All colonies 2010 (n=8)	0.215 (0.094-0.353)	0	-0.126, (-0.428-0.069)	0.0004	0.385 (0.212-0.543)

Simple family colonies 2010 (n=2)	0.195	0	-0.296	0.0005	0.408
	(-0.004- 0.398)		(-0.635- 0.048)		(-0.012-0.683)
Mixed family colonies 2010 (n=5)	0.245	0	-0.106	0.0095	0.421
	(0.111- 0.392)		(-0.412- 0.124)		(0.236- 0.589)
Simple family colonies Fall/Winter 2010 (n=8)	0.217	0	-0.858	0	0.797
	(0.220-0.072)		(-0.918--0.778)		(0.645-0.863)
All colonies 2011 (n=8)	0.120 (0.036-0.241)	0	-0.221 (-0.5-0.042)	0	0.259 (0.119-0.436)
Simple family colonies 2011 (n=2)	0.184	0	-0.282	0	0.387
	(0.009- 0.371)		(-0.571- 0.080)		(0.032- 0.538)
Mixed family colonies 2011 (n=1)	0.121	0	-0.199	0	0.256
	(0.038-0.256)		(-0.521-0.095)		(0.119-0.467)

Table 2: F-statistics: *Reticulitermes flavipes* worker relatedness estimates (r) in natural colonies (Confidence intervals of 95% are shown in parentheses and the sample size (n) refers to the number of colonies studied in each population). P-values were estimated by permutations.

A negative FIC value in May 2009 (FIC=-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 presented levels of genetic differentiation among the colonies FCT (0.441). In May 2010, the overall measure of inbreeding, FIT 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 FIC (-0.296) indicating an heterozygote excess compared to a panmictic population with the same allele frequencies. This is consistent with the expected value of FIC for a simple family (-0.209 to -0.33) [9,14]. Furthermore, simple family colonies were genetically different with positive FCT value (FCT=0.195) (Table 2). Mixed family colonies also had a negative FIC (-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 (FCT=0.245) (Table 2). In early November 2010, all the colonies changed back into simple family colony type with negative FIC (FIC=-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 FCT 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 FIC value (FIC=-0.221). Low negative FIC values (FIC=-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 FCT value (FCT=0.184), which lower than in previous years. Mixed family colonies also show medium excess heterozygosity with negative FIC value (FIC=-0.199) with moderate genetic differentiation among colonies (FCT=0.121) (Table 2). The low relatedness values in May 2011 suggested that positive and assortive mating might occur.

Discussion

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 [19,23]. In early literature on inbreeding and relatedness in termites, [26] suggested that the life histories of the ancestral termites may have been characterized by alternate inbreeding and outbreeding generations.

Bartz [26] proposed that the overwintering generation die off before the spring generation starts to reproduce. These offspring cannot become workers in the parental nest but they can mate and produce a summer generation and are assumed to live to the end of the season. Under this strategy, a termite has two alternatives, either helping the parent to raise more offspring, or to mate and produce their own offspring. If they produce their own offspring, they will outbreed and produce outbred offspring. Therefore, the summer generation will be more related to their sibling than they would be with their own offspring [19,26]. In addition, when the founding pair is unable to reproduce or die, then the development of multiple secondary reproductives occurs [17,27,28].

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 [7,8,29-31]. 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. These data support the earlier literature cited above. 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 reproductives [5,7,27,29-32]

Studies of other species, there was variation among populations of the European species, *R. grassei*, suggesting large numbers of neotenics in some populations and low numbers in others. In the North American Reticulitermes species, extended families in most populations contained relatively few reproductives, and these were likely the direct offspring of the founding pair [8,17]. These conclusions were similar to the results from laboratory colonies in which the reproductive composition was determined to be at low level genetic relatedness [33]. Laboratory studies of *R. flavipes* have shown that neotenics may sometimes coexist with primary reproductives [33,34]. A primary king together with 25 female neotenics in the African subterranean termite, *Schedorhinotermes lamanianus*, [29] demonstrating that primary and neotenic reproductives of other species do sometimes occur within the same colony in the field.

According to our data, 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 and established a new termite colony. 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 [12,22,35]. Colony fusion defines as when two unrelated individuals from two separate colonies breed together to form mixed colonies [12]. Furthermore, the stability of mixed family colonies over the long term is unclear. The field data [22] and lab [36] indicated that the presence of multiple unrelated groups of reproductives in fused colonies for *R. flavpies* is generally short lived, which are similar to this study.

[6] 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, [22] 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 [22]. In this study, a very high level of genetic differentiation occurred among all eight colonies over the three years 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 [29]. 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 [17,29]. In this study, intraspecific patterns of variation in breeding systems over three years varied from the same collection point.

Another study on *R. flavipes* indicated that colony foraging areas can change radically when some colonies are eliminated through chemical means [11]. This suggests that in these habitats, it is not a question of whether foragers come in contact with one another that determines the likelihood of forming mixed family colonies, but rather how they interact when their foraging territories share common borders [28,37]. Other hypotheses explaining the merging of unrelated colonies in social insects in nature may invoke changes in odor-cue diversity [38] or a reduction in social motivation caused by queenlessness [39] to explain the fusion of unrelated groups.

In addition, *R. flavipes* is a native termites in Nebraska and most of eastern United States, colonies structure showed a major change for the period of three year. This similar to *R. flavipes* colonies in France also showed major quantitative changes in social structure and can be compared to super colonies of ants in that they allow individuals to mix freely between colonies [4,40]. Due to a reduced diversity at recognition alleles through a genetic bottleneck as proposed in invasive ants, social organization of colonies could have evolved after the introduction into a new environment [38] or from a selection process against less common alleles [41]

In other insect groups like Hymenoptera, changes in social organization pattern and movements have already been observed as in the case of the wasp, *Vespula germanica* [42,43] and the bumblebee, *Bombus terrestris* [44,45]. In ants, movement and changes in social organization are still unclear due to the unicolonial and multicolonial organization patterns as described in the native range of several species of ants [46-49]. A hypothesis that occasional "mixing" of non-nestmates may occur at foraging sites but without necessarily compromising the genetic boundaries of the colonies, and thus not automatically resulting in mixed colonies. Furthermore, according to [50], insect societies are always closed according to sociobiological theories due to food sharing behavior and the observed existence of inter-colonial agonism. Therefore, a strong territoriality is generally assumed in termites due to the location of the study is in the natural population where the food resources are widely available and make the termite territorial looking for food is wide. However, the agonistic behavior of some *Reticulitermes spp.* is known to vary in individual colony combinations [51,52] (Thorne and Haverty, Polizzi and Forschler) and also shows some seasonality [53,54]. Invading the space of another colony is to capture or share food resources can be the obvious reason for the agonistic behavior [49].

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